ANTIOXIDANT ROLES
OF URIC ACID AND TYROSINE
IN MAMMALIAN ERYTHROCYTES

by

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ABSTRACT

Various works have shown the presence of transport mechanisms in erythrocytes, which facilitate the transport of tyrosine and urate across erythrocytes membranes. In his study on mammalian erythrocyte pathophysiology [22], B. Weber observed that high amounts of tyrosine were present in Black rhinoceros erythrocytes, and that uric acid could, on occasion, be identified in erythrocyte extracts of other species (human, horse, and cow). This latter observation came as a surprise since it is known that mammalian erythrocytes lack the machinery to metabolise urate. Although an antioxidant role for tyrosine could be proposed from the experimental results obtained by B. Weber, no clear explanation could be formulated to justify the presence of urate within erythrocytes.

The aim of this study was to investigate the mechanisms through which urate and tyrosine are metabolised in human and horse erythrocytes, and to establish their roles within erythrocytes.

Most of the analyses were performed using reverse-phase High Performance Liquid Chromatography (HPLC), for which erythrocytes were prepared using a Perchloric acid extraction protocol. The antioxidant abilities of certain compounds were measured using an ORAC (oxygen radical absorbance capacity) assay, and later, a cell culture experimental system was set up to assess the protective effect of erythrocytes on fibroblasts exposed to free radicals.

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Upon analysis of race horse erythrocyte extracts, unexpected pre- and post-exercise dynamics of urate levels were observed. The urate level patterns appeared to be influenced by oxidative stress, but they were not fully consistent over short periods of time (i.e. 24 hrs – 72 hrs). A 9week-analysis, however, showed, with consistency, that exercise could induce a significant increase of urate levels in horse erythrocytes.

With ORAC assays, it could be demonstrated that urate and tyrosine were both able to behave as antioxidants towards peroxyl radicals generated by AAPH. The antioxidant capacity of urate, in particular, seemed to be preserved within horse erythrocyte extracts, which suggested that urate in horse erythrocytes may be able to act towards scavenging free radicals produced during exercise.

Urate was observed to disappear within 24hrs of erythrocyte extraction in human erythrocyte extracts, which made it difficult to perform experiments on human extracts over a long period of time. An investigation of this phenomenon revealed that temperature strongly influenced urate levels within whole erythrocytes and erythrocyte extracts. The reasons for urate instability in human erythrocytes were unclear, however, it was suspected that haem, which, through various studies, was shown to possess catalytic capacities [18, 22], was a likely candidate responsible for the disappearance of urate from erythrocyte extracts. In the presence of either haem or H_2O_2 alone urate was not significantly degraded. However, in the presence of both haem and H_2O_2 urate was rapidly converted to at least three products. The elution times and absorption spectrum between these products and allantoin were very close, indicating that these molecules might have structural similarities.

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The degradation of urate caused by haem, in the presence of H_2O_2, showed similarities with the uricase reaction. Surprisingly, the allantoin produced in the latter reaction did not have a structure identical to that of an allantoin marker.

The addition of haem, with or without H_2O_2, to the urate precursors hypoxanthine and xanthine, produced no observable effects.

Other oxidants were tested to compare their effects to that of H_2O_2. Experiments showed that HOCl degraded urate but with no observable products on HPLC, and that AAPH showed no observable effect on urate, which seemed to contradict results obtained during the ORAC assays, where an interaction between urate and peroxyl radicals, generated by AAPH, could be shown.

Urate and tyrosine transport was investigated in human and horse erythrocytes by monitoring urate- and tyrosine-peak area variations in erythrocyte extract. The erythrocyte uptake of these two compounds was successfully demonstrated in human erythrocytes but not in horse erythrocytes. The uptake of urate and tyrosine could be linked to antioxidant mechanisms, allowing the compounds to enter the erythrocytes' intracellular environment to scavenge free radicals accumulated during oxidative stress.

Fibroblast culture experiments showed some evidence of protection of fibroblasts by erythrocytes against peroxyl radicals. It could not be established, however, whether the efficiency of erythrocytes in protecting against oxidative damage was species-specific. Urate- and tyrosine-containing

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erythrocytes did not show better protection of fibroblasts compared to other erythrocytes.

This study has, then, highlighted the antioxidant roles of uric acid and tyrosine, emphasising urate metabolism, which could be associated with antioxidant mechanisms . The possibility of urate being able to play a protective role for erythrocytes, and other tissues has been mentioned.

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To the Maker of all things.

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ABBREVIATIONS

μCi: micro Curie

3-NT: 3-Nitrotyrosine

AAPH: 2,2'-Azobis(2-methylpropionamidine) dihydrochloride

AD: Alzheimer's Disease

AMPS: Ammonium Persulfate

BPE: B-phycoerythrin

dH_2O: Distilled water

EAE: Experimental Allergic Encephalomyelitis

GSH: Glutathione

H_2O_2: Hydrogen Peroxide

HD: Huntington's Disease

HOCl: Hypochlorous Acid

HPLC: High Performance Liquid Chromatography

Hx: Hypoxanthine

Ino: Inosine

iNOS: Inducible Nitric Oxide Synthase

K_2CO_3: Potassium Chromate

LN_2: Liquid Nitrogen

MetHb: Methaemoglobin

MS: Multiple Sclerosis

$NO^{\bullet}$: Nitic Oxide

$O_2^{\bullet}$: Superoxide Radical

$OH^{\bullet}$: Hydroxyl free radical

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ONOO : Peroxynitrite

PBS: Phosphate Buffer Saline

PCA: Perchloric Acid

PD: Parkinson's Disease

RBC: Erythrocytes

SOD: Superoxide Dismutase

TCA: Trichloro-Acetic acid

Trolox: 6-hydoxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

UA: Uric Acid

Xa: Xanthine

XO: Xanthine Oxidase

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Chapter 1: INTRODUCTION

1.1 Background:

Erythrocytes are well known for their ability to transport oxygen and carbon dioxide across the organism. Their function is not limited to this however; erythrocytes are also able to transport, across their plasma membranes, various other substances such as amino acids, water, nucleosides and sugars [23]. These compounds are transported across erythrocyte membranes through mechanisms that are coordinated and regulated by many erythrocyte-enzymatic systems and ion pumps. Tyrosine transport, for instance, is dependent on a protein structure intrinsic to human erythrocyte membranes, which relies upon the involvement of sulfhydryl groups (these are thiol groups that are essential in maintaining the structural integrity of the erythrocyte membrane) [23]. Three transport systems for amino acids have been described in human erythrocytes:

- The L-system, which is the major pathway for large neutral amino acids [19].
- The Ly$^+$-system for dibasic amino acids [19].
- The T-system, which is directed towards aromatic amino acids [19].

Studies have shown that tyrosine uptake occurs by both the L- and the T-systems [19].

Erythrocytes have also been shown to be involved in uric acid transport. For instance, in 1930, Folin and Svedberg identified the presence of uric acid in human erythrocytes [32], and in 1961, U.V. Lassen demonstrated urate influx and efflux across erythrocyte membranes [18]. Lassen's study showed that the kinetics of uric acid transport are similar to the kinetics of most active

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transport systems, which rules out any possibility for passive diffusion of urate across erythrocyte membranes. This suggests that uric acid enters human erythrocytes through a "facilitated" or "equilibrating carrier" mechanism [18]. However, this mechanism has yet to be fully elucidated. Lassen performed experiments which consisted of observing whether the transport of urate was dependent upon the pH of the medium used to suspend the erythrocytes. He managed to show that urate is transported more rapidly at low pH values, which indicates that uric acid is transported through the erythrocyte membrane in the acid (undissociated) form [18].

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Interest in erythrocyte urate and tyrosine began when B.W. Weber observed the presence of tyrosine in black rhinoceros erythrocytes [22], in amounts that greatly exceeded those of most other mammalian erythrocytes. The results of further investigations suggested that tyrosine plays an antioxidant role, and might contribute towards protecting the erythrocytes against hemolysis [22]. During the course of his study, Weber also noted that, when extracts from other mammalian erythrocytes were analysed by High Performance Liquid Chromatography (HPLC), an unknown species was occasionally observed in HPLC traces. This compound was later identified as uric acid. Levels of uric acid, however, varied greatly across species, and even within the same species, though generally the levels were higher in the horse. The reasons for this, and the role of uric acid in the erythrocytes, remained unclear.

The aims of this project are

- To investigate the metabolism of uric acid (or urate) and tyrosine in human and horse erythrocytes.

- To test the hypothesis that this might play a role in specific antioxidant mechanisms that can protect the erythrocyte itself, or other tissues in the erythrocyte's microenvironment against free radicals.

1.2 Free radicals and oxidants:

Free radicals are chemical species with unpaired electrons that are often, but not always, highly reactive, and that usually have a transient existence. Because of their high reactivity, free radicals are able to cause damage to most major cellular macromolecules such as DNA, proteins, lipids, and lipoproteins. However, many aerobic systems utilise metabolic processes that are based on free radical reactions (for example: photosynthesis), and the reaction products of these metabolic processes can be dangerous if not properly controlled.

Free radicals are not only formed as by-products of aerobic metabolism. They are also formed through the action of environmental agents, and as cyto-toxic weapons of the immune system and of inflammatory responses. For instance, activated neutrophils and macrophages generate large fluxes of superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2). Also, macrophages produce nitric oxide (NO), through the inducible enzyme nitric oxide synthase (iNOS) [1].

Reactive oxygen species (ROS), which form a major category of free radicals, are products of reactions involved in the reduction of dioxygen (O_2). The complete reduction of O_2 involves the transfer of 4 electrons per oxygen molecule to generate the oxide O^{2-} ion, which has a stable electronic configuration. Intermediate levels of reduction are, however, possible:

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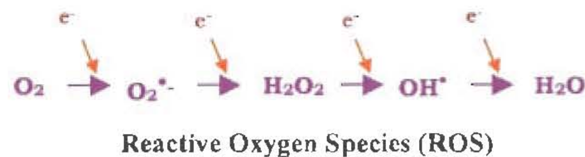
- The one-electron reduction of O_2 produces the superoxide free radical anion $O_2^{\bullet -}$.
- Further reduction produces the peroxide ion O_2^{2-}, which in the protonated form can bind to a hydrogen molecule to form hydrogen peroxide (H_2O_2).
- In the presence of oxidisable transition metal ions, such as iron and copper, peroxides are broken down to the hydroxyl free radical $OH^{\bullet}$. $OH^{\bullet}$ is highly reactive and is able to abstract a hydrogen atom from a wide range of organic compounds.



Reactive Oxygen Species (ROS)

These three reduced oxygen products (i.e. $O_2^{\bullet -}$, H_2O_2, and $OH^{\bullet}$) constitute the Reactive Oxygen Species (ROS).

All reactions in the bioenergetics scheme are completely dependent on the transfer of electrons to dioxygen [2]. However, about 98% of the oxygen metabolised is handled by the mitochondrial enzyme cytochrome oxidase alone. This enzyme transfers 4 electrons to oxygen in a concerted reaction to produce two molecules of water [2]. However, 1% to 2% of the total electron flux through the mitochondria leaks out through at least two sites in the electron-transport chain: complex I and ubisemiquinone. The electrons leak out to waiting oxygen molecules, resulting in the formation of $O_2^{\bullet -}$.

The free radical nitric oxide ($NO^{\bullet}$) is generated in biological tissues and is an important regulator of a wide range of biological functions [3]. Recently, it was recognised as a signalling molecule [4] which plays a critical role in modulating vascular tone. However, $NO^{\bullet}$ also has cytotoxic and genotoxic effects through its ability to damage proteins and DNA. It destroys the iron-

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sulfur centres of key enzymes such as aconitase, which has an important role in cellular energy production. $NO^{\bullet}$ can also damage DNA by nitrosative deamination of DNA bases, leading to the formation of mutagenic products [4]. Nitric oxide can combine with $O_2^{\bullet}$ to form peroxynitrite ($ONOO^-$), which induces various damaging oxidative processes including lipid peroxidation [5].

1.3 Oxidative damage and disease:

The excessive production or insufficient disposal of ROS constitutes oxidative stress. Some of the various exogenous stresses that may cause an oxidative wave are:

- Energetic radiation, which can generate $OH^{\bullet}$ and singlet oxygen.
- Increase in oxygen supply (hyperoxia), which may increase the natural formation of ROS.
- Stimulation of the immune system, which may lead to a massive local production of ROS and HOCl (hypochlorous acid, the strongest physiological oxidant) because of the activities of NADPH oxidase and myeloperoxidase in the phagocytes.
- Some xenobiotics can also boost the production of ROS [6].

Oxidative stress contributes to neuronal death in a range of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington disease (HD), and Multiple sclerosis (MS) [7]. In PD, for instance, the pathogenic process, which is characterised by progressive akinesia, tremor and rigidity, involves mitochondrial dysfunction and increased levels of oxidative stress [8]. The latter may arise from the metabolism of dopamine, leading to the autoxidation of dopamine itself. This autoxidation leads to the production of semiquinones, which in turn lead to the generation of ROS [9].

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Free radical damage to proteins, lipoproteins and DNA can also play a role in the pathology of autoimmune diseases such as rheumatoid arthritis, systemic lupus erythemathosus, insulin dependent diabetes mellitus and atherosclerosis [10]. In these autoimmune diseases free radicals seem to be key factors in perpetuating the chronicity of inflammation and in producing potential auto-antigens through biomolecule denaturation.
Other diseases in which free radicals are agents of pathogenesis are: chronic tissue rejection, cardiomyopathy, septic shock, and post-ischemic injury.

1.4 Antioxidant mechanisms:

In order to avoid the damaging consequences of uncontrolled free radical reactions, all aerobic organisms have developed an array of protective antioxidant mechanisms. Enzymatic antioxidant mechanisms constitute the majority of antioxidant mechanisms, eliminating undesirable toxic species by means of catalysis. Families of antioxidant enzymes include:

- Superoxide dismutases for the elimination of superoxide radicals.
- Catalases and glutathione peroxidases for the elimination of hydrogen peroxide [6].

In addition to antioxidant enzymes, there are a number of low molecular mass substances, which can play an important role in controlling free radical processes in biological systems. These substances are agents of the non-enzymatic antioxidant mechanisms and include ascorbate, glutathione, tocopherol, ubiquinol, ergothioneine, hypotaurine, lipoic acid, and uric acid [10].

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1.5 **Uric acid as an antioxidant:**

Uric acid (or urate) is a naturally occurring product of purine metabolism known to effectively scavenge peroxynitrite ($ONOO^-$), and accounting for 30-65% of the total peroxyl radical-scavenging ability of human plasma [12]. The scavenging property of urate was investigated by Hooper *et al.* [11] in a series of experiments performed on mice that developed a chronic form of Experimental Allergic Encephalomyelitis (EAE), which produces symptoms that are similar to Multiple sclerosis in humans (MS). Both diseases are characterised by peroxynitrite-induced lipid peroxidation in the central nervous system [11]). In his study, Hooper administered various doses of uric acid to affected mice at different time intervals and found that uric acid had strong therapeutic effects in a dose dependent manner, and suggested that urate has an inhibitory effect on peroxynitrite-induced lipid peroxidation in EAE, thus implying that urate could play a role in slowing down or inhibiting the spread or production of CNS plaques in MS patients [11]. An observation consistent with these results is that patients with MS have significantly lower levels of serum uric acid than healthy subjects. Also, hyperuricemia and MS seem to be mutually exclusive.

A study on pneumoccocal meningitis showed that urate was able to attenuate meningeal inflammation, blood-brain barrier disruption, and intra-cranial hypertension by scavenging $ONOO^-$ [27].

In addition to scavenging $ONOO^-$, urate is also able to scavenge singlet oxygen (which is a lipid peroxidation initiator as well), and hydroxyl radicals generated from H_2O_2 by either $FeSO_4$ or UV light [13]. Uric acid also suppresses lipid peroxidation in erythrocytes, slowing down the process of erythrocyte ageing [13].

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1.6 Tyrosine:

When cells are exposed to oxidative stress, proteins become oxidatively modified and then selectively degraded. This proteolytic response to oxidative stress occurs in many cell types, including erythrocytes. *In vitro* studies of protein modification by oxygen radicals and related oxidants showed the formation of covalent bonds between tyrosine residues to form dityrosine [14] during oxidative stress. The formation of dityrosine has been attributed mainly to the activation of the myeloperoxidase/H_2O_2 antimicrobial system of neutrophils and macrophages, but reactions with peroxynitrite and other peroxidases have been recognised as additional sources of dityrosine. Dityrosine, then, can be used as a specific marker for free radical modification of proteins and protein oxidation in cells. It has already been identified in human atherosclerosis plaques, in patients affected with Alzheimer's disease, in age-related nuclear cataract and other pathologies thought to be associated with oxidative stress [4].

The dityrosine molecule contains a phenolic group, which may confer protective abilities to the molecule since various phenolic compounds have been shown to be able to scavenge superoxide ions and to suppress the production of lipid peroxides [15].

Tyrosine was also conferred protective abilities when it was observed, in *Saccaromyces cerevisiae*, that impairment in the capacity to synthesise tyrosine, or loss of the TYR 1 gene function (involved in the production of tyrosine), resulted in high sensitivity towards peroxide [15]. Also, through a method measuring the oxidant capacities of several compounds present in the seminal fluid (which is known to contains various non-enzymatic ROS

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scavengers), researchers could establish that tyrosine is one of the most important contributors to its total antioxidant capacity [25].

1.7 Erythrocytes

Erythrocyte membranes are permeable to exogenous ROS like H_2O_2 and $O_2^{\bullet}$. However, they contain large amounts of antioxidants such as catalase, glutathione, and superoxide dismutase, which enable them to deactivate ROS that diffuse through their membranes. These enzymes, together, account for the majority of ROS-scavenging activity of erythrocytes [16].
A number of studies have suggested that erythrocytes can scavenge H_2O_2 within their microenvironment, such as the H_2O_2 generated by neutrophils during immune responses. Through this function, erythrocytes are able to prevent the formation of $OH^{\bullet}$ and HOCl in the blood [16]. By eliminating extracellular H_2O_2, erythrocytes are able to cause a decrease H_2O_2 present within the neutrophils, and this in turn leads to an inhibition of neutrophil apoptosis [16]. Similarly, erythrocytes are able to inhibit the T-cell apoptotic process, started after mitogenic activation of resting human peripheral blood T-cells [17] in a process which also involves ROS scavenging.
Hence, erythrocytes have the potential to constitute a major antioxidant system that is able, for example, to suppress neutrophil and T cell apoptosis in the blood. It has not yet been reported whether other cells and surrounding tissues can also be protected by erythrocytes against free radical-mediated damage.

In this study, attempts are made to define the protective role of tyrosine and urate in mammalian erythrocytes, and to see whether these roles are

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consistent with a more general protective role for erythrocytes in protecting peripheral tissues.

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Chapter 2: MATERIALS and METHODS

2.1 Source of blood samples

Blood samples were mainly collected from horse and human subjects, because these samples were more readily available than samples from other species.

Horse blood samples were obtained from the Blue Cross Veterinary Hospital and from horse owners at racing events, while human blood samples were obtained from healthy subjects in the department of Chemical Pathology.

2.2 Preparation of blood samples

Blood samples were collected from subjects into sterile vac-u-test heparin-lithium tubes. The samples were centrifuged for 10min at 3500rpm, in a Sigma 350X centrifuge, in order to separate erythrocytes from plasma and buffy coat. The erythrocytes were then washed three times in an isotonic solution of 0.9% NaCl (9g/l), and used for experiments.

2.3 Freezing of erythrocytes

When collected blood samples were not intended for immediate use the separated erythrocytes were frozen down in liquid Nitrogen as follows: washed erythrocytes were diluted 1:1 in a freezing medium (450mM Sucrose, 325mM Glucose, 50mM NaCl). The mixture was then frozen dropwise in liquid nitrogen (liqN_2) and then stored at -70°C. Erythrocytes could be kept in these conditions for several months.

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2.4 High Performance Liquid Chromatography (HPLC) analysis

2.4.1 Erythrocyte preparation for HPLC

In preparation for HPLC analysis (described in section 2.4.2) washed erythrocytes were suspended to one volume of saline and two volumes of ice cold 1.2M Perchloric acid (PCA – Merck) to precipitate erythrocyte proteins. PCA was added while vigorously mixing erythrocyte with saline on a Vortex-genie2 vortex mixer (Scientific Industries Inc., New York, USA). erythrocyte-proteins were then precipitated by centrifuging the extracted samples in an ultracentrifuge (Lasec, Pty. Ltd.) for 20 seconds. The supernatant was collected and neutralised with 2.5M K_2CO_3 (BCH) to pH > 7. Crystals of potassium perchlorate, which formed during neutralisation, were precipitated by ultracentrifugation for 10 sec. Extracts (referred to as erythrocyte extracts) were then injected onto a reverse-phase-HPLC column for analysis.

2.4.2 HPLC analysis

Erythrocyte extracts were analysed by Reverse Phase HPLC. The HPLC system consisted of a Beckman 126 programmable solvent module connected to a 168-diode array detector. The reverse-phase column used for separations was a 250X4.6mm (5μm) ODS column (Hypersil). After extensive use, this column was replaced by a 250X4.6mm Ultrasphere C18 (5μm) column (Beckman Instruments Inc., San Ramon, California, USA).
The buffers required for HPLC analysis were 10mM KH_2PO_4 (BDH Laboratory supplies) at pH 5.6 (buffer A) and 50% Methanol (Riedel-de-Haen, Methanol for HPLC) (buffer B). Buffers were made up in deionised water, filtered and then degassed.

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Initial conditions for HPLC analysis were 1% buffer B and 99% buffer A. After 5min these conditions were gradually changed over 3min to 20% buffer B and 80% buffer A. Conditions were maintained as such for 15min, then returned to initial settings over another 3min. The total running time for analysis was 26min10sec [22].

A volume of 100μl of each sample to be analysed (50μl for human samples) was injected into the column, and UV absorbance was monitored at 260nm and at 280nm. When allantoin detection was required, wavelengths were set at 220nm and 280nm (this is because the maximum absorbance of allantoin occurs in the region of 220nm and 225nm). Compounds were identified by their retention time, 260/280 ratio (or 220/280 ratio), co-elution with equivalent standards and maximum absorbance [22].

2.4.3 Standards:

In order to determine tyrosine and urate concentrations in the samples analysed by HPLC, the following procedure was followed:

- Solutions of uric acid and tyrosine were made up at various concentrations: Uric acid: **12.5μM, 25μM, 50μM**
  Tyrosine: **25μM, 50μM, 100μM, 200μM, 400μM**
- Each solution was analysed on HPLC, and HPLC profiles from each analysis were used to evaluate urate or tyrosine peak areas.
- Standard graphs were obtained by plotting the peak area of the compound in each solution against the known concentration.

Figure 1 shows the graphs that were obtained.

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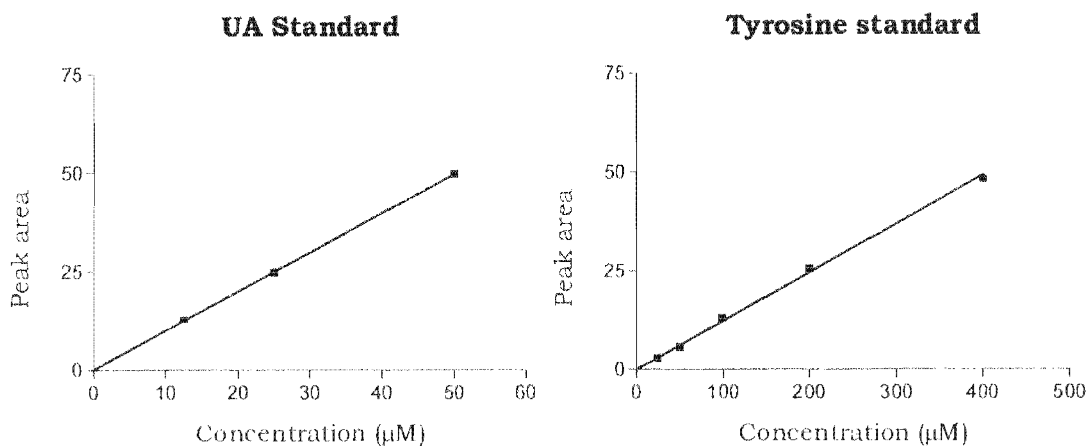

FIGURE 1: Standard graphs of uric acid and tyrosine.

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When concentrations needed to be deduced from erythrocyte HPLC profiles, the dilution factor of 1:4 (introduced with the addition of PCA and saline to packed erythrocytes) needed to be taken into consideration. Hence, peak area values obtained from HPLC traces were multiplied by 4 to obtain the approximate concentrations (in μM) of tyrosine or urate present in intact erythrocytes.

2.5 Oxygen Radical Absorbance Capacity (ORAC) assay

2.5.1 Reagents and assay set-up

Antioxidant capacities of urate, tyrosine, and erythrocyte extracts were evaluated using an ORAC assay [20]. This assay utilises the free radical-sensitive fluorescent reporter-protein Beta-phycoerythrin (BPE) to monitor the effectiveness of assayed samples in protecting BPE from becoming damaged by free radicals. The reagents used in ORAC assay mixtures were:

- B-phycoerythrin (BPE, Sigma).

- 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH, Aldrich Chemical Company, Inc.), which is a peroxyl radical generator.
- 6-hydroxy-2,5,7,8-tetramethylchroman-2,2-carboxylic acid (or Trolox, Aldrich Chemical Company, Inc.), a known antioxidant used here as a positive control [27].

The BPE solution was made up in PBS buffer by diluting BPE in the following proportions:

96µl BPE (0.5mg stock solution) **in 10.5ml PBS**

The BPE solution was ready for use after a 15-min incubation period at 37°C.

ORAC assays were performed using an Aminco, SPF-500 Fluorometer, and reaction mixtures were set up in glass cuvettes as shown in table 1 below.

Table 1: ORAC assay reagents and their proportions in the reaction mixture.
* The term "Sample" refers to either of the following:
- ERYTHROCYTE extracts diluted 1:20 or 1:100 in PBS
- Urate or tyrosine solution, 12.5µM.

Reagents	Blank	Trolox	Sample
Phosphate buffer, 0.75M	200µl	100µl	-
Trolox, 100µM	-	100µl	-
Sample *	-	-	200µl
B-PE, 0.04µM	1600µl	1600µl	1600µl
AAPH, 40mM	200µl	200µl	200µl

Reagents were added to a final volume of 2 ml in 10mm wide glass cuvettes, and reactions were started with the addition of AAPH.

Fluorescence was measured every 10min at the emission and excitation wavelengths of 565nm and 540nm respectively.

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2.5.2 Calculations:

The peroxyl radical absorbing ability of a fluid is given by the number of micromoles of peroxyl radical trapped by 1 litre of fluid. The ORAC value refers to the net protection area under the BPE curve (AUC) in the presence of plasma or trolox or other reagents, minus the blank, which is performed with phosphate buffer [20].

The ORAC value is calculated using the formula below. This formula uses a stoichiometric factor of 2 for trolox because 1μmol of trolox is able to trap 2μmol of peroxyl radicals [20].

ORAC value (μmol/l) = **2k X (S sample – S blank) / [(S trolox – S blank)/5]**

Where:
"k" is the dilution factor;
"2" is the trolox stoichiometric factor;
"S" is the area under the BPE curve (AUC) in the presence of the sample or trolox;
"5" is the final concentration of trolox (in μM).

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2.6 Haem catalysis experiments

In vitro experiments were performed to assess the catalytic properties of haem on urate in the presence of H_2O_2. A stock solution of 1mM hemin (bovine, from Sigma) was prepared in 20mM NaOH (Sarchem), and was added to urate and H_2O_2 to a final concentration of 10μM. Urate and H_2O_2 were used at concentrations of 100μM and 2mM respectively. Experimental samples were incubated at 37°C for a few seconds, then run on reverse phase HPLC for analysis. Control reactions did not contain either hemin or H_2O_2.

2.7 Transport studies

Transport experiments were conducted with a hematocrit of 10-20%, in one of the following incubation buffers depending on the experiment conducted:

- PBS-glucose (140mM NaCl, 7mM K_2HPO_4, 2.5mM KH_2PO_4, 10mM glucose) [28].
- "Tyrosine-uptake buffer" (140mM NaCl, 5mM KCl, 2mM $MgCl_2$, 15mM Tris) [19].
- Sucrose buffer (260mM sucrose, 20mM hepes) [21]. This buffer was used specifically for urate uptake studies to facilitate urate uptake in erythrocytes.
- Hanks balanced salt solution [26].

Each buffer contained either 0.5mM urate or 1mM tyrosine, and pH was adjusted to 7.4.

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2.8 Tissue culture experiments:

Tissue culture experiments were performed in order to investigate the capacities of urate, tyrosine and erythrocytes in protecting cultured human skin fibroblasts against various oxidants.

Cultured fibroblasts were trypsinised (with 0.125% in 0.5mmol/l trypsin, made up in phosphate buffer) and plated onto 24-welled plates with fresh medium:

- DMEM with Na Pyruvate, 1000mg/l glucose with pyridoxine (Highveld Biological (PTY) Ltd.) or
- DMEM with 4.5g/l glucose without glutamine (Gibco, Bio-Whittaker) + Glutamine (L-Glutamine 200mM, Highveld Biological (PTY) LTD, RSA)].

Cells were allowed to settle overnight, and medium was washed off the following day with Hanks balanced salt solution. This was performed in preparation for an incubation step of fibroblasts in Hanks solution containing AAPH in the presence of radioactive labels: 1μCi/ml ^{3}H-

Phenylalanine and 0.2μCi/ml ^{14}C-uridine. Both labels were used together in the presence of the oxidants to assess whether RNA synthesis or protein synthesis were efficient ways to monitor cell damage.

After a 4hr-incubation period of fibroblast cells at 37°C (in the presence of the labels and the oxidant), unincorporated labels were washed off three times with trichloro-acetic acid (TCA, B&M Scientific). Fibroblasts were then dissolved with 250μl of 0.1M NaOH (per well), and aliquoted into scintillation vials containing 5ml of liquid scintillation cocktail (Ready Flow III, Beckman). Lastly, label incorporation was measured in a Liquid Scintillation Spectrometer (1600 TR, Packard).

When erythrocytes needed to be included in the experiments, a 1:100 solution of packed erythrocytes was made up in Hanks, and added to the fibroblasts at different amounts (i.e. 1μl and 10μl). Before analysis, the erythrocytes were washed off, along with the unincorporated labels, with TCA.

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Chapter 3: RESULTS

3.1 Urate dynamics in racehorse erythrocytes:

During the initial stages of this study, erythrocyte extracts from the following mammalian species, human, horse, rhino, and cow, were analysed by HPLC. Each sample was collected from animals at rest, and it was observed that some of the samples contained urate and/or tyrosine, while other samples did not.

Most horse HPLC profiles showed a significant peak of tyrosine. The figure below shows a typical HPLC trace illustrating this:

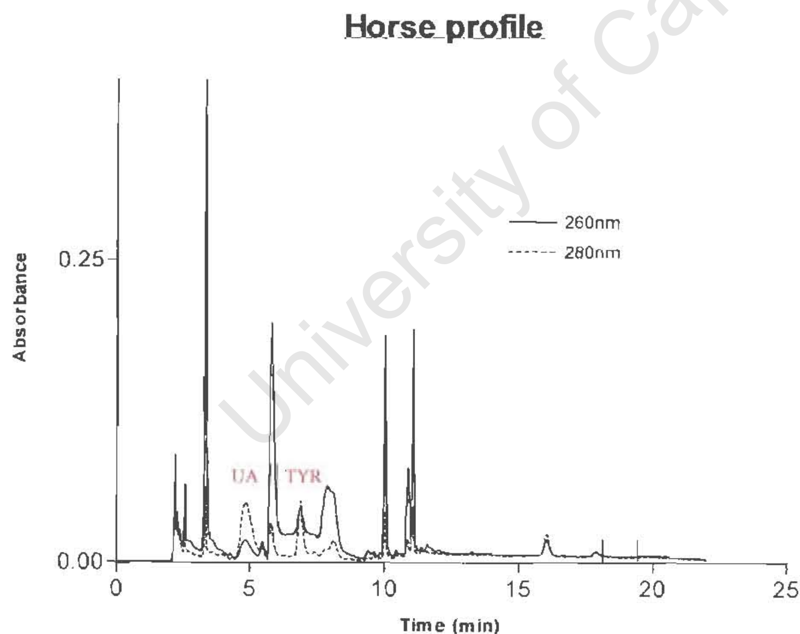

FIGURE 2: Typical horse HPLC profile showing both a Tyrosine and a Uric acid peak (as indicated in red letters). **UA:** Uric acid, **TYR:** tyrosine

The average amount of tyrosine present in the horse erythrocytes that were analysed was approximately 200μM.

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The levels of tyrosine and uric acid varied across species and even within the same species. This is illustrated in figure 3 below, where representative HPLC traces of horse and human erythrocytes are shown.

A study on exercise-induced physiological variations in human blood, conducted by Ames *et al.* [13], led to the observation that plasma urate levels increased with exercise. This level increase was thought to be due to a marked inhibition of renal clearance of urate by lactate and β-hydroxybutyrate during exercise resulting in an accumulation of urate in the blood. Ames suggested that the urate level increase occurred as part of a physiological mechanism that helps the body to cope with increased oxidative stress induced by exercise [13].

An opportunity to investigate whether this phenomenon could also be observed in race horses presented itself when horse blood samples, collected during racing events, were analysed on HPLC.

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3.1.1 <u>First sampling:</u>

Blood samples were collected from 8 horses (A, B, C, D, F, G, H, and J) during an 80km-race at the following timepoints:

- T_1: 24^h before the race
- T_2: Immediately after the race
- T_3: 1^h after the race
- T_4: 24^h after the race
- T_5: 72^h after the race

After blood sample collection, erythrocytes were separated from plasma and buffy coat by centrifugation, washed three times in saline, extracted with perchloric acid (PCA), then neutralised with K_2CO_3 to pH 7-9 before analysis on reverse-phase HPLC (Materials and Methods, section 2.4). Urate peak areas were obtained from HPLC analysis data, and were used to deduce the urate concentration in each sample using a urate standard graph (shown in

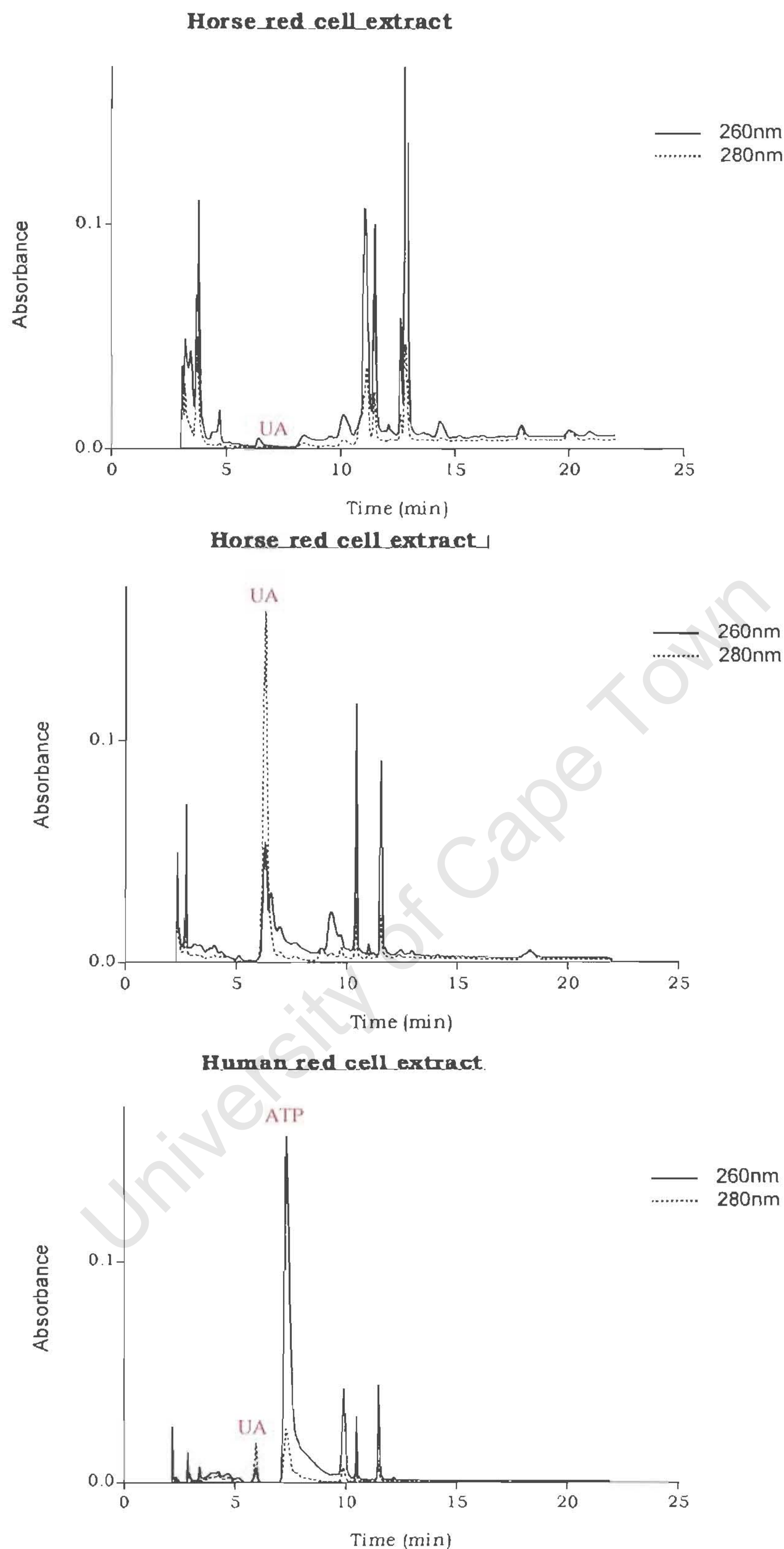

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FIGURE 3: HPLC profiles of human and horse red cell extracts. Horse and human samples were injected into the HPLC column in volumes of 100µl and 50µl respectively. ATP levels were higher in human because the sample was analysed shortly after blood collection, whereas horse samples were analysed days after blood collection.

figure 1 section 2.5).

Urate concentration values obtained from the analysis of horse cell extracts are given in the table below:

Table 2: First sampling: Urate concentrations (in µM) in horse erythrocytes. T_1: 24hrs before the race, T_2: Immediately after the race, T_3: 1hr after the race, T_4: 24hrs after the race, and T_5: 72hrs after the race.
The symbol * is used to indicate samples that were not collected because horse owners were unavailable or unwilling for blood samples to be taken from their horse(s).

	A	B	C	D	F	G	H	J
T1	56.4	0.0	0.0	0.0	67.6	0.0	0.0	1.7
T2	118.4	0.0	422.0	7.6	0.0	0.0	0.0	2.0
T3	108.8	0.0	824.0	35.2	157.6	0.0	32.4	35.5
T4	0.0	0.0	658.4	11.6	*	0.0	0.0	42.6
T5	160.0	0.0	393.6	148.0	0.0	0.0	125.6	31.0

In this table one can see that, of the eight sample series analysed, only six showed clear urate level variations in relation to the time.

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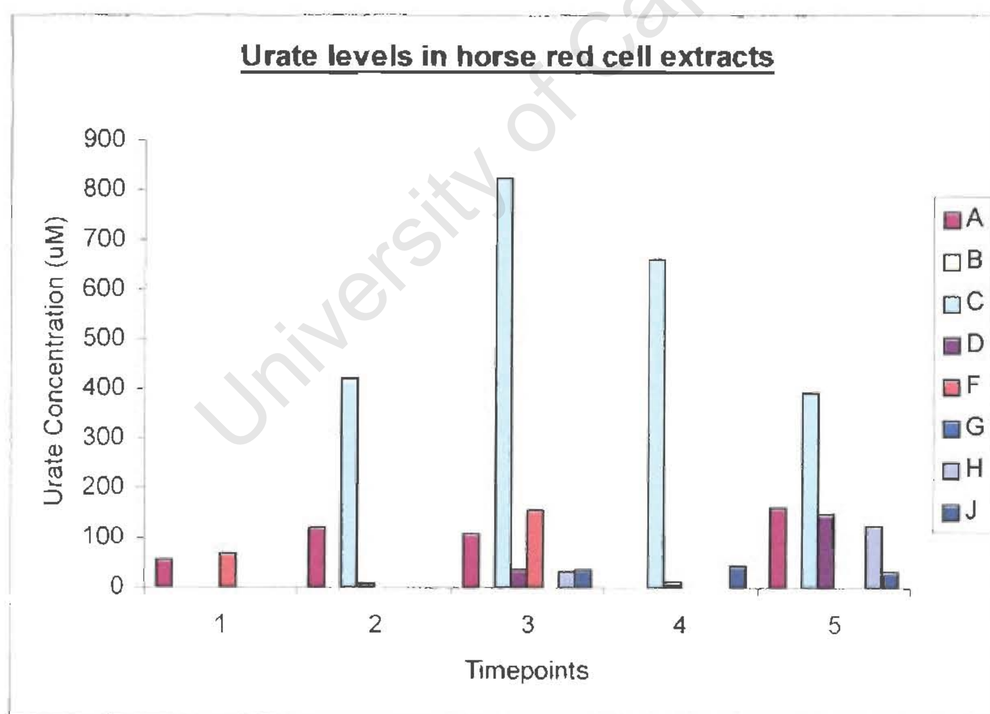

FIGURE 4: Histogram of urate concentration in the horse red cells at the different timepoints. Concentrations are in µM and timepoints are as follows: T_1 - T_5 are defined in Table 2.

As figure 4 shows, urate level variation patterns differed from one horse to the next. For instance, only horse J samples showed urate at all timepoints. Horses B and G showed no urate at any of the timepoints. In general, urate seemed to appear sporadically. However, the presence of urate in the extracts was more frequent at timepoints 3 and 5. Also, at these timepoints, most samples showed higher levels of urate than at either T_1, T_2 or T_4.
The urate levels for horse C were strikingly higher (approximately 10-fold higher) than the urate levels of the other horses. This may have been due to greater effort on the part of horse C which showed a higher level of strain after the race, compared to the other horses (as confirmed by measurements of various parameters such as glucose concentration, white blood cell counts, plasma volume, cortisol concentration, etc. - P. Robson, personal communications).

3.1.2 <u>Second sampling:</u>

In an attempt to attach a statistical significance to the dynamics of urate levels observed in the previous experiment, a bigger sample size was chosen for the second blood sampling, which took place under similar conditions to the first one (i.e. same distance and similar weather conditions). This time, blood samples were collected from 12 horses (A, B, C, D, E, F, H, I, J, K, L, and X) at the following timepoints:

- T_1: 24^h before the race
- T_2: Immediately after the race
- T_3: 1^h after the race

Erythrocytes were prepared for HPLC analysis (Materials and Methods, section 2.4), and urate concentrations were deduced from the urate peaks areas. Urate concentration values obtained were the following:

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Table 3: Second sampling: Urate concentrations (in μM) in horse erythrocytes. T_1: 24hrs before the race, T_2: Immediately after the race, and T_3: 1hr after the race.

	A	B	C	D	E	F	H	I	J	K	L	X
T1	47.0	144.2	0.0	0.0	73.4	0.0	0.0	0.0	0.0	0.0	0.0	13.0
T2	0.0	99.0	0.0	0.0	120.4	0.0	0.0	0.0	0.0	0.0	0.0	25.3
T3	12.3	165.2	0.0	0.0	103.8	0.0	0.0	0.0	0.0	0.0	0.0	62.2

Figure 5 shows that only 4 of the 12 horses showed significant amounts of urate (A, B, E and X), and 3 out of those 4 samples showed urate at all three timepoints (B, E and X). No consistent trend was thus observed in these horses.

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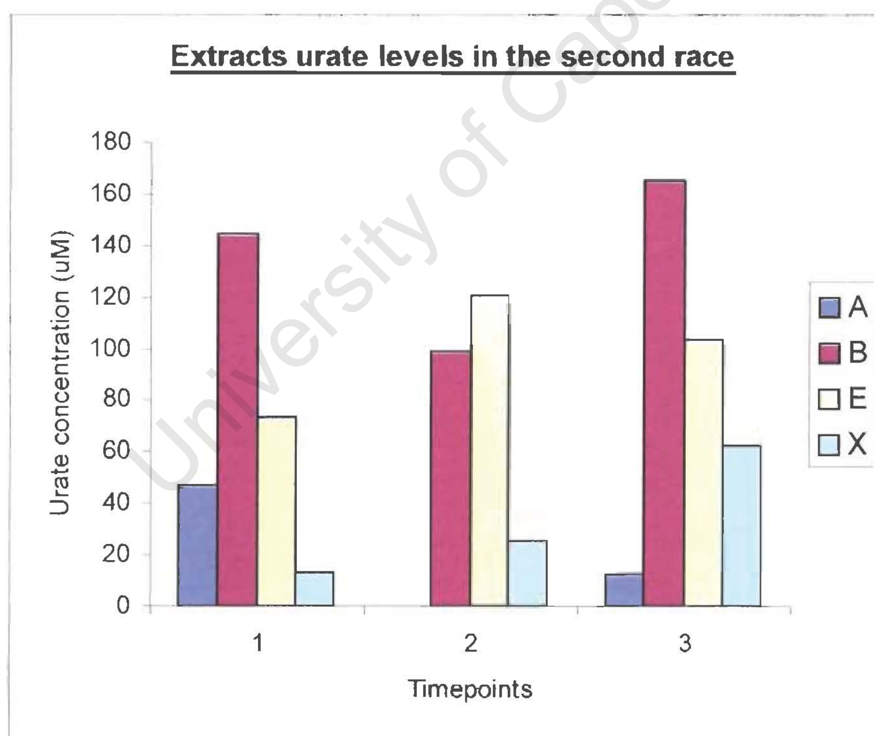

FIGURE 5: Histogram of urate concentration (μM) in horse erythrocytes during the second sampling.
T_1 - T_3 are defined in Table 3.

3.1.3 **Third sampling:**

In the hope to obtain more consistent patterns of urate level variations, the the timepoints for blood sampling of this third sampling (which took place during a 9-week training period) were set over a longer period of time:

- T_1: Before training
- T_2: After 9 weeks of training

The urate concentrations values that were obtained after HPLC analysis were the following:

Table 4: Third sampling: Horse erythrocyte urate concentrations (μM) over a 9-week training. T_1: before training, and T_2: after 9 weeks of training.

	A	B	C	D	E	F	G	H
T1	32.8	0.0	0.0	0.0	17.2	114.4	0.0	0.0
T2	30.0	0.0	15.4	178.2	113.8	176.8	186.4	141.2

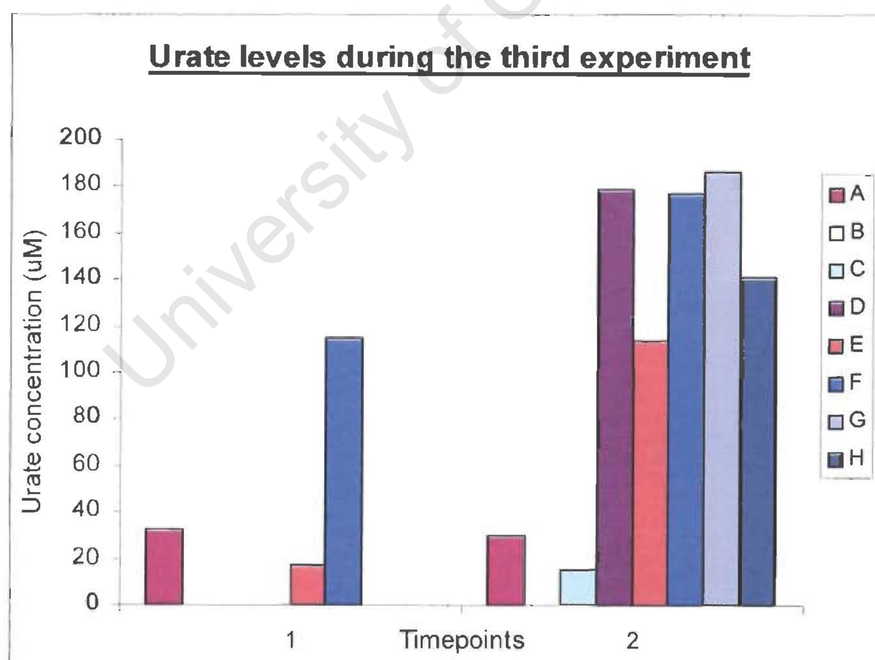

FIGURE 6: Urate concentrations (μM) in horse red cells over a 9-week training.
T_1 and T_2 are defined in Table 4.

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As figure 6 illustrates, urate was already present in 3 of the horses (horses A, E and F) before exercise. After a 9-week training period, all horses showed an increase in the erythrocyte urate concentration (with the exception of horse A, in which urate levels did not vary much). Horse B, however, showed no trace of urate at any of the timepoints.

The statistical significance of these results was evaluated using a statistical simulation performed using TRUE BASIC. The algorithm designed for this test was the following:

```
CLEAR
Input prompt "How many individuals measured?": N
RANDOMIZE
LET CC,CCC,CCCC=0
FOR J=1 TO 10000
        LET C=0
        FOR K=1 TO N
                IF RND>.5 THEN LET C=C+1
        NEXT K
        IF C=N THEN LET CC=CC+1
        IF C=N-1 THEN LET CCC=CCC+1
        IF C=N-2 THEN LET CCCC=CCCC+1
NEXT J
PRINT
PRINT "Frequency of";N;"out of ";N;"=";cc/100;"%"
PRINT "Frequency of";N-1;"out of ";N;"=";ccc/100;"%"
PRINT "Frequency of";N-2;"out of ";N;"=";cccc/100;"%"
END
```

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Since only 7 of the 8 horses showed any trace of uric acid, the number N was set at 7. According to this simulation, the chances for uric acid levels to increase between timepoint T_1 and timepoint T_2:

(a) in 7 out of 7 horses was 0.90%
(b) in 6 out of 7 horses was 5.01%
(c) in 5 out of 7 horses was 16.72%

This simulation shows that the likelihood for urate levels to increase in 6 of 7 horses is 5.01%, approaching the determining value of 5% for statistical significance. However, a bigger sample size is needed in order to obtain more reliable results.

These experiments have highlighted the presence of urate within horse erythrocytes and its level variations after exercise.
The increase of urate levels in horse erythrocytes could confirm observations made by Ames *et al.* in their physiological study on human blood, which had led them to conclude that increased urate in human blood plasma could be consistent with a process in which physical exercise induces the production or transport of urate in the blood [13].

A pilot study was undertaken to investigate whether the same phenomenon also occurred in human erythrocytes. Blood was collected from a human subject before, immediately after and 1hr after a Mountain biking exercise. Changes in urate levels were, however, not significant (as shown in figure 7 below). Only a slight decrease in urate levels occurred between the first two timepoints (figure 7).

In summary, horse erythrocytes showed unexpected dynamics of urate levels during and after exercise. Although these urate levels appeared to be influenced by exercise, the patterns were not fully consistent or reproducible over a short period of time (i.e. 24 hrs – 72 hrs). However, a long-term analysis (9 weeks) revealed that exercise could induce a significant increase of urate levels in horse erythrocytes. The elevated amounts of urate in erythrocytes after exercise may indicate that either urate plays an antioxidant role protecting erythrocytes against free radicals accumulated during exercise, or the presence of urate in the erythrocyte may be a reflection of antioxidant activities occurring in other tissues such as muscle tissue, in which case erythrocyte urate could be used as a marker for oxidative stress.

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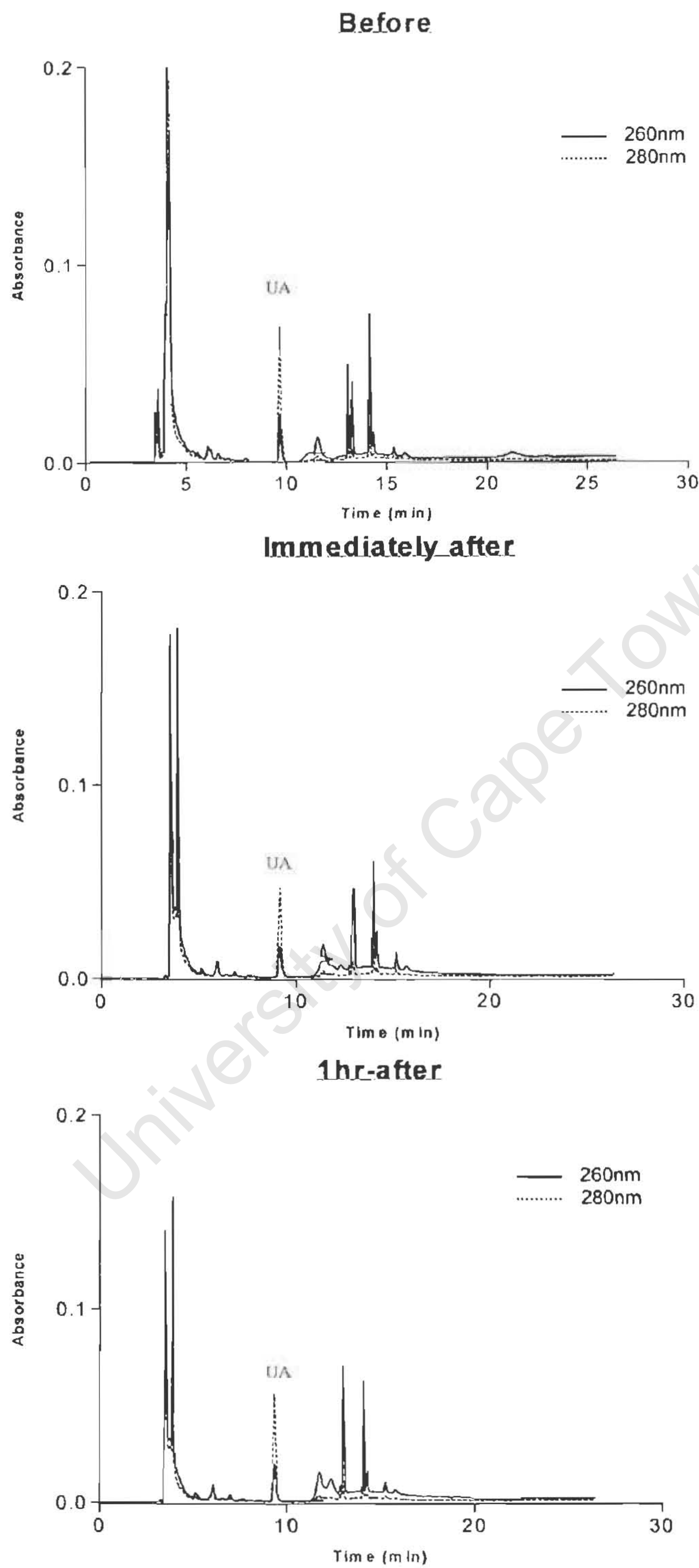

FIGURE 7: Pilot study of urate dynamics during exercise on a human subject.

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3.2 Urate and Tyrosine antioxidant capacities

To investigate the antioxidant capacities of urate and tyrosine, the antioxidant capacities of these two compounds were assessed using an ORAC assay [20,27] (Materials and Method, section 2.5.1), which looked at their abilities in protecting the reporter protein B-phycoerythrin (BPE) against peroxyl radicals generated by 2,2'-Azobis (2-methylpropionamidine) dihydrochloride (AAPH).

Urate and tyrosine were added to reaction mixtures (as described in Table 1) containing BPE and AAPH, to a final concentration of 12.5μM. BPE-absorbance was measured every 10min using a fluorometer (Aminco, SPF-500), and absorbance readings were then plotted on a graph (as shown in figure 8 below). A decrease in absorbance indicates BPE damage by AAPH. The "blank"-curve decreased very rapidly, resulting in an Area Under the Curve (AUC) of 223.4. However, the trolox curve (a known antioxidant used here as a positive control) showed a plateau for the first 60 minutes of the reaction, indicating a strong ability to protect the BPE-protein against peroxyl radicals. After this initial 60min-period, the graph decreased over approximately 2hrs, indicating that the trolox was being used up resulting in a decrease of its protection of BPE.

The urate-graph, like the trolox-graph, showed a plateau for the first 50 minutes, indicating strong antioxidant abilities and a high affinity interaction between urate and the peroxyl radicals generated by AAPH. Tyrosine did not show a plateau, but protected BPE for a longer period of time than urate.

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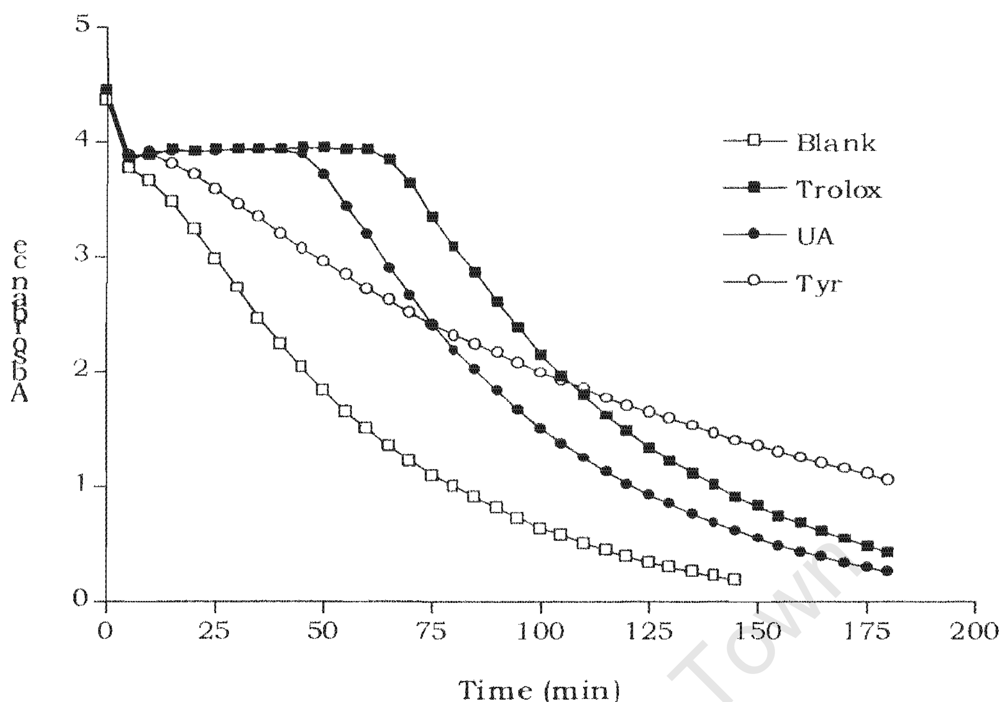

FIGURE 8: Time dependent fluorescence decay of BPE after addition of AAPH in the presence of: **Blank**: plain PBS, **Trolox**: 5μM Trolox, **UA**: 12.5μM urate, and **Tyr**: 12.5μM tyrosine.

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The area under the curve (AUC) and the ORAC value of each substance were calculated using the formula given in section 2.5.2. Table 5 shows the values obtained from the calculations.

Table 5: Area under the curve (AUC) and ORAC values for urate and tyrosine obtained from the results in figure 6.

	Blank	Trolox	Urate	Tyrosine
Area Under the Curve	223.4	449.2	381.1	419.0
ORAC value (μmol/l)	-	-	**7.0**	**8.7**

Tyrosine and urate produced similar overall ORAC values (8.7μmol/l and 7.0μmol/l respectively), which indicates that these two compounds have similar antioxidant capacities.

In summary, results of this ORAC assay have shown that urate and tyrosine are both able to behave as antioxidants towards peroxyl radicals generated by AAPH, and that their interactions with these free radicals differ.

3.3 Erythrocyte extract antioxidant capacities:

In order to see whether urate retains its ability to scavenge free radicals within horse erythrocyte extracts, ORAC assays were performed on two horse erythrocyte extracts, obtained from blood samples collected during the first horse race: one which contained urate (Extract-A_3: Horse A at T_3, 108.8μM urate) and one which did not show any urate (Extract-B_3: Horse B at T_3, 0μM urate).

The plotted data obtained from the ORAC assay of these two extracts are

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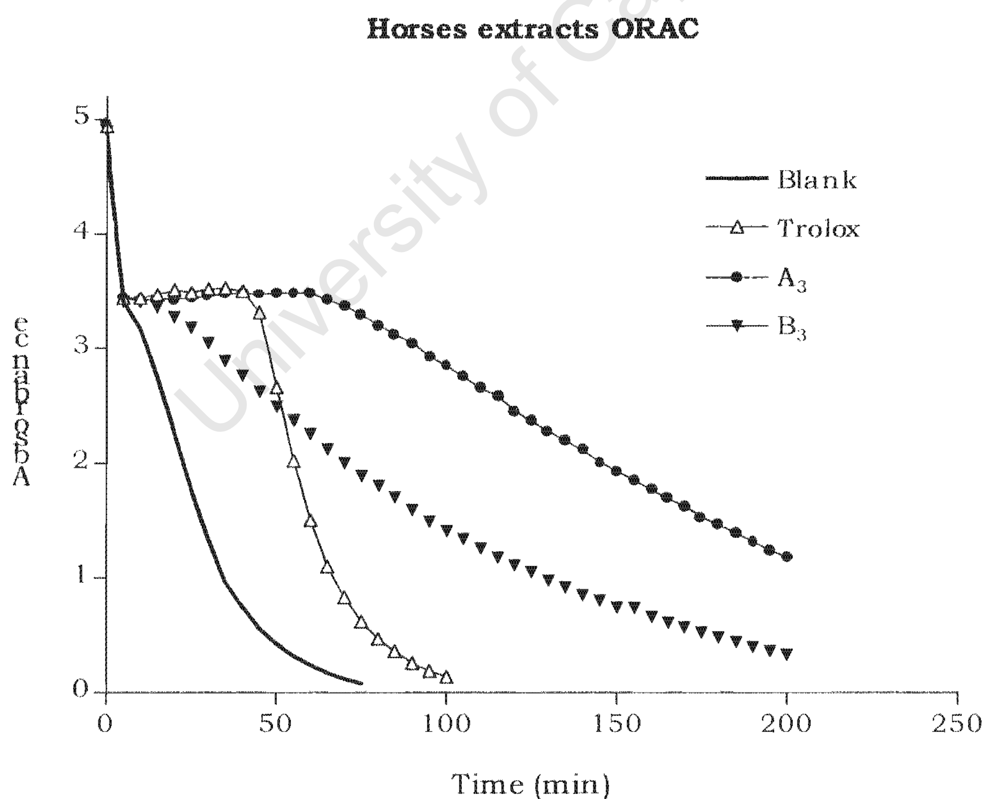

FIGURE 9: Graphs of antioxidant capacities of horse extracts **A_3** (108.8μM urate) and **B_3** (0μM urate) against a **Blank** (PBS), and **Trolox** (positive control). RBC-extracts were diluted in PBS (1:20), and then added into the reaction cuvettes as described in Materials and Methods (section 2.5).

shown in figure 9 above.

The A_3-graph showed a plateau similar to that of the urate-graph in figure 8, and ORAC values obtained from the graphs were A_3: 747.8μmol/l and B_3: 398.8μmol/l. These results indicate a stronger ability for A_3 to inhibit peroxyl radicals as opposed to B_3. The high antioxidant capacity of the A_3-extract may be attributed to the presence of urate in this extract, which agrees with the hypothesis that urate in horse erythrocytes can act to scavenge the free radicals produced during exercise.

3.4 Urate stability in erythrocyte extracts:

During this study, blood samples that were collected from subjects were either immediately extracted and analysed, or extracted then stored at 4°C until they could be analysed. It was observed that urate could remain stable for many days (even weeks) in horse erythrocyte extracts during storage. However, urate levels decreased markedly and even disappeared within 24hrs of erythrocyte extraction in human erythrocyte extracts. This made it difficult to perform experiments on human extracts over a long period of time.

It was not clear why urate was more stable in horse extracts than in human extracts, so attempts were made to investigate whether storage temperature played any role in affecting the stability of urate in human extracts. For this, a series of experiments were performed, the details of which are given in figure 10 below.

Freshly collected human blood (from a healthy subject) was centrifuged to separate erythrocytes from plasma, and, after a few washes in saline, erythrocytes were incubated in PBS-glucose for different periods of time at 0°C, 25°C, and 37°C. Cell extraction and HPLC analysis were done at various

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timepoints, and urate stability was compared between whole erythrocytes and in PCA extracts over time (see representative scheme of the experiment in figure 10).

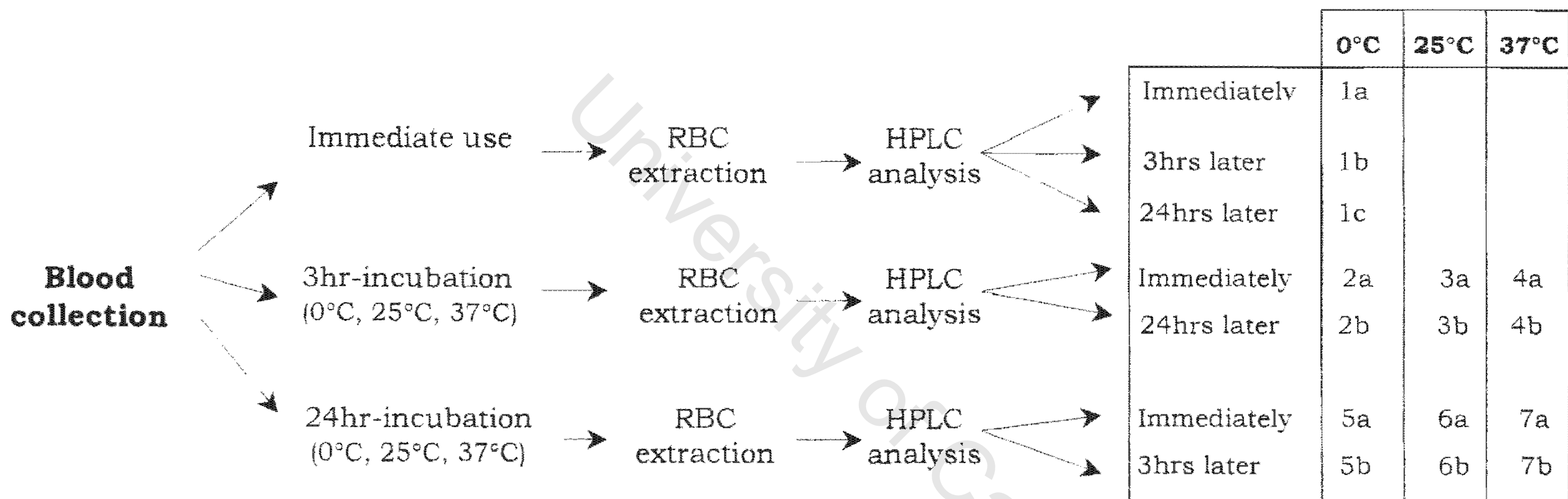

FIGURE 10: Scheme followed for the Temperature experiments.
The labels *1a, 2a,* etc... refer to different experimental tubes. The same numbering is used for the corresponding graphs below (Figures 8 and 9). Samples **2**, **3**, and **4** could not be analysed after 3hrs because the experiments were logistically difficult to organise (similarly, samples **5**, **6**, and **7** could not be analysed after 24hrs).

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HPLC profiles of representative samples of this experiment are given in figure 11 below. One can see from the graphs that uric acid levels were lower in 2a than in 1a (2a was extracted and analysed after an incubation on ice for 3hrs, as opposed to 1a which was extracted and analysed immediately after blood collection); the urate level decrease from 1a to 2a is likely to have occurred during the 3hr-period of incubation of erythrocytes on ice.

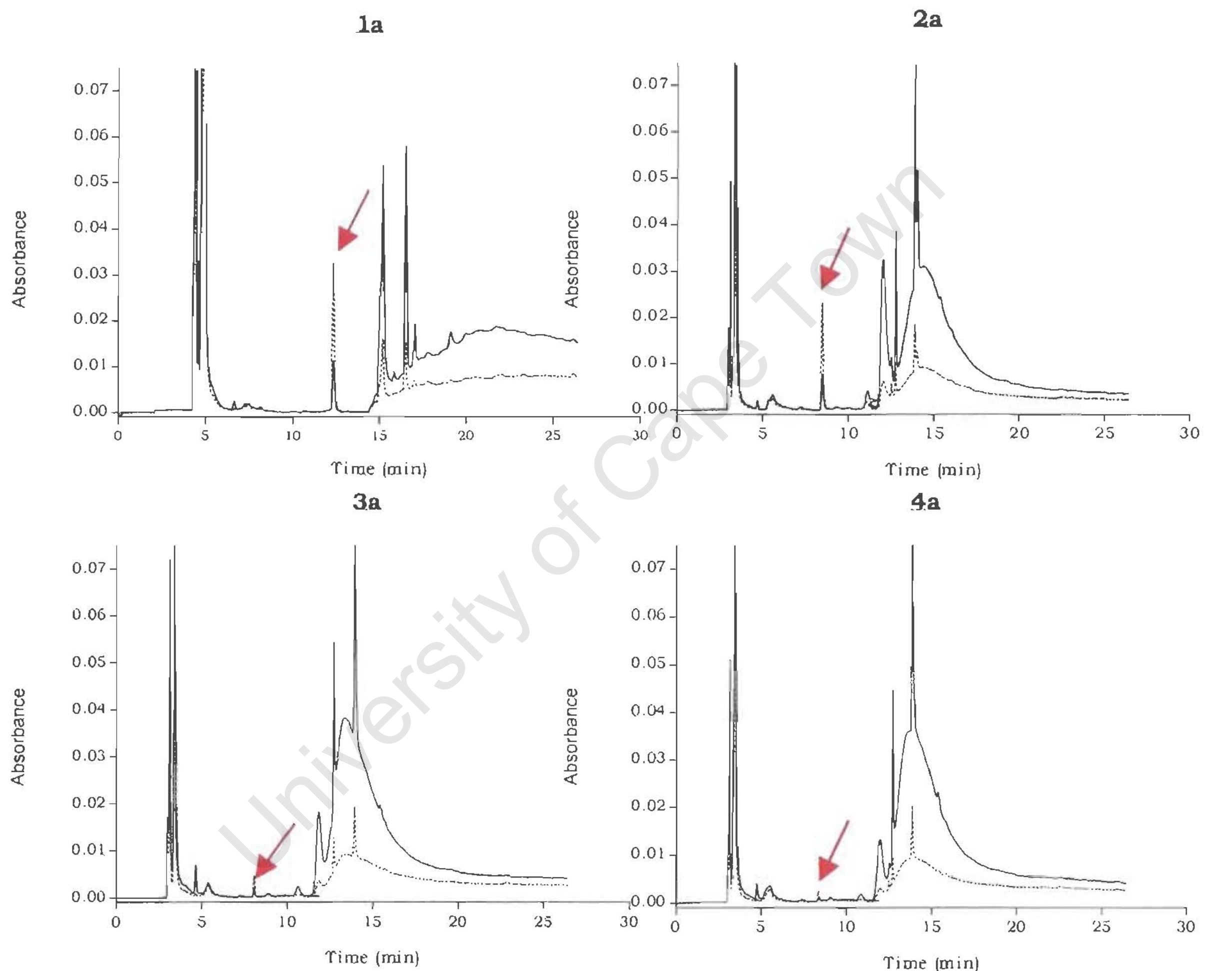

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FIGURE 11: HPLC profiles of representative samples. The urate peaks displaying the characteristic 280/260nm absorbance properties are indicated with a red arrow.

............: Absorbance read at 280nm

———: Absorbance read at 260nm

Erythrocytes in samples 3a and 4a were incubated for 3hrs at 25°C and 37°C, respectively. They were then extracted and analysed immediately after incubation. The marked decrease of urate level is quite apparent in these

two samples, and it occurred faster at 37°C than at 25°C, strongly indicating that urate is unstable in erythrocytes at temperatures higher than 0°C. The histograms below illustrate this better.

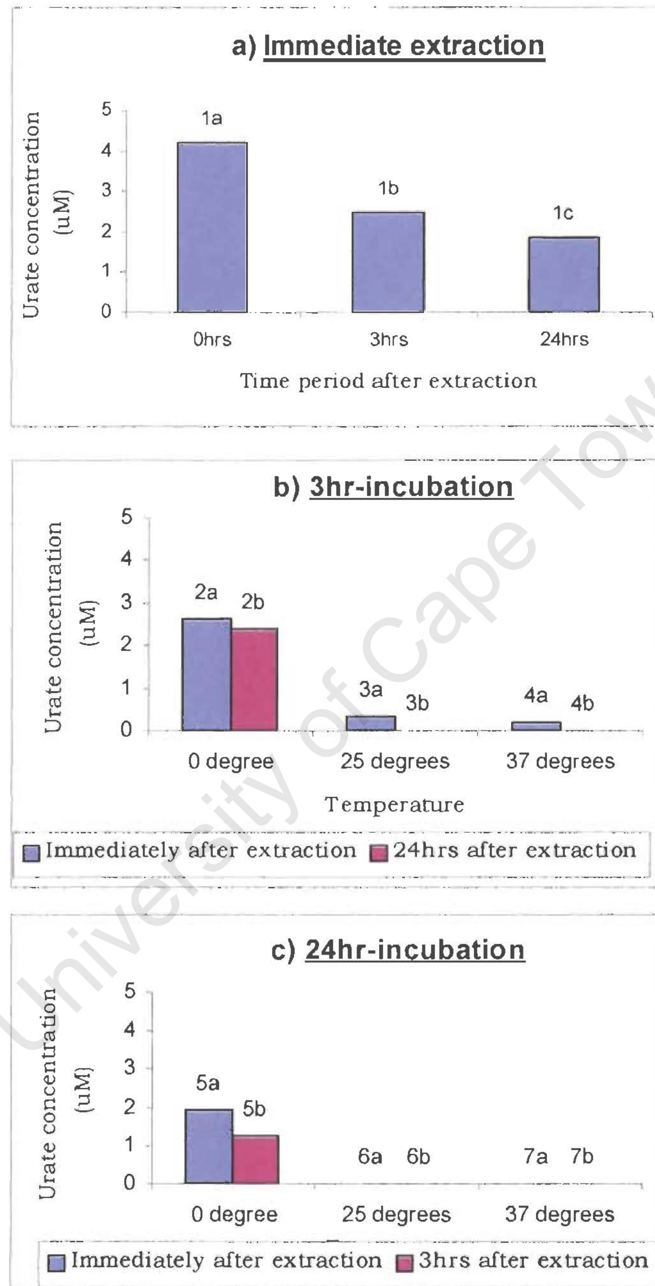

FIGURE 12: Histograms of urate concentrations in the temperature experiments. Sample labels indicate experiments described in figure 9.

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Results shown in figure 12a indicate that over a 24hr-period urate levels decreased by about 50% (observe urate level variations between samples 1a to 1c), pointing out the unstable nature of urate within erythrocyte extracts at low temperatures.

Figure 12b shows that urate levels present in the erythrocytes (as shown in sample 1a), decreased by about 40% after a 3hr-incubation of these cells on ice (shown in sample 2a). Urate levels decreased even faster during incubations of whole erythrocytes at higher temperatures; for example, only 10% of the original amount of urate (sample 1a) was present in the cells after incubation at 25°C (sample 3a), and less than 5% remained in the cells after incubation at 37°C (sample 4a). Extracted samples 3a and 4a were kept overnight at 4°C and analysed on HPLC one more time the following day to see whether the urate levels would vary overnight. This second analysis showed that urate had disappeared completely from the two extracts during the overnight storage (samples 3b and 4b, figure 12b).

In figure 12c, one can see that urate levels did not change dramatically in whole erythrocytes when they were incubated for 24hrs on ice (compare 2a-urate levels to 5a-urate levels). However, erythrocyte incubation at 25°C and 37°C caused urate to disappear completely from the samples (samples 6a and 7a).

In summary Figures 11 and 12, together, strongly indicate that uric acid is very unstable in human erythrocytes and that storage conditions markedly influence its levels within whole erythrocytes and erythrocyte extracts.

The reasons for this instability are not yet clear, however a starting process to investigate this was to determine whether extracts contained residual

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erythrocyte component (such as haem) which may have contributed to the instability of urate in the extracts.

3.5 Catabolic effects of Haem and H_2O_2:

3.5.1 Catabolic effects on urate:

Erythrocyte extracts contain no proteins (hence no enzymes) because these are precipitated during PCA extraction (Materials and Methods, section 2.4.1). Extracts, however, contain mainly ions and small molecules such as haem, which has been shown to participate in antioxidant mechanisms by catalysing the formation of dityrosine from tyrosine in the presence of hydrogen peroxide [22]. Since urate degradation, which has been identified in the previous experiments of this study, could be a part of an erythrocyte-antioxidant mechanism, and because various studies have shown that haem possesses catalytic capacities [18, 22], the latter seemed a likely candidate responsible for the disappearance of urate from erythrocyte extracts in antioxidative processes.

To investigate and to identify any role that haem might play in urate degradation, *in vitro* experiments were set up as shown in table 6.:

Table 6: Experimental setup for urate catalysing reactions

Mixture	Urate (25μM)	Haem (10μM)	H_2O_2 (2mM)	Allantoin (100μM)
a	+	-	-	-
b	+	+	+	-
c	-	-	-	+
d	+	+	+	+

Reagents, which included urate (25μM), haem (10μM), allantoin (100μM), H_2O_2 (2mM) and uricase (2μg/ml), were mixed in dH_2O, and allowed to react for 40 seconds before they were analysed on HPLC.

Figure 13 shows some of the results that were obtained.

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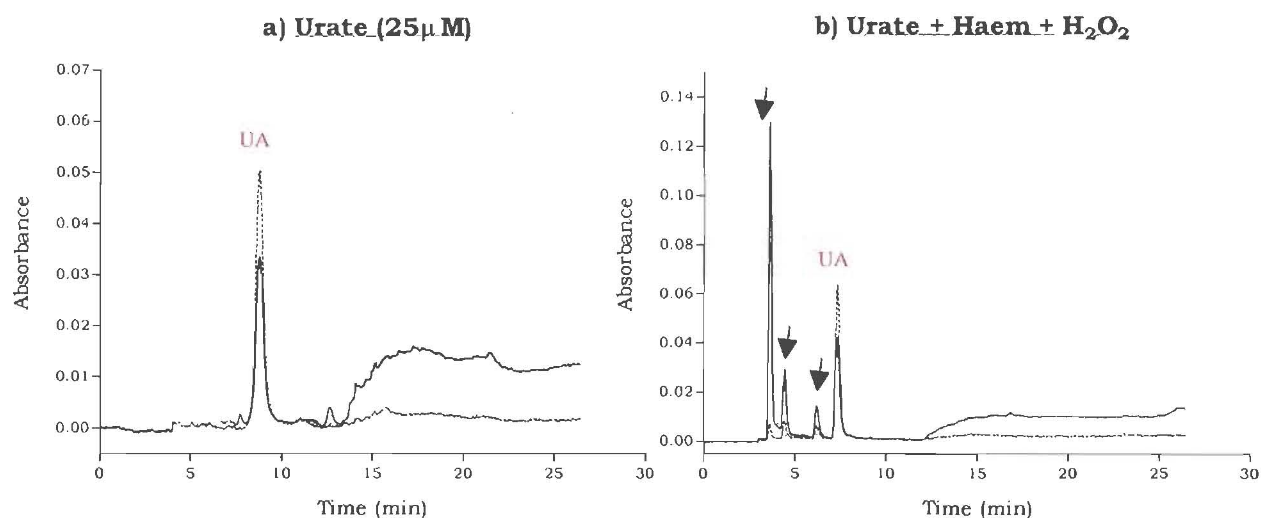

FIGURE 13: HPLC profiles of the uric acid catalysing reaction **a:** control (25μM UA), **b:** uric acid (25μM), haem (10μM), and H_2O_2 (2mM).
Urate products are indicated by arrow heads in figure 13b.
..............: Absorbance read at 280nm
————: Absorbance read at 220nm

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In the presence of either haem or H_2O_2 alone urate is not significantly degraded (data not shown). However, in the presence of both haem and H_2O_2 urate is rapidly converted to at least three products (figure 13b).

To see whether one of the urate products generated during this reaction was allantoin, the reaction in 1b was performed in the presence of allantoin, used here as a marker (100μM allantoin). Because allantoin has maximum absorbance between 220-230nm, the wavelengths used for HPLC analysis of the allantoin-containing samples were set at 220nm and 280nm. As seen in figure 14d, the allantoin marker (indicated by a red arrow) was found to elute between the first and the second urate product. The elution times and absorption spectrum between the products and allantoin are close and may indicate that these molecules have some structural similarities.

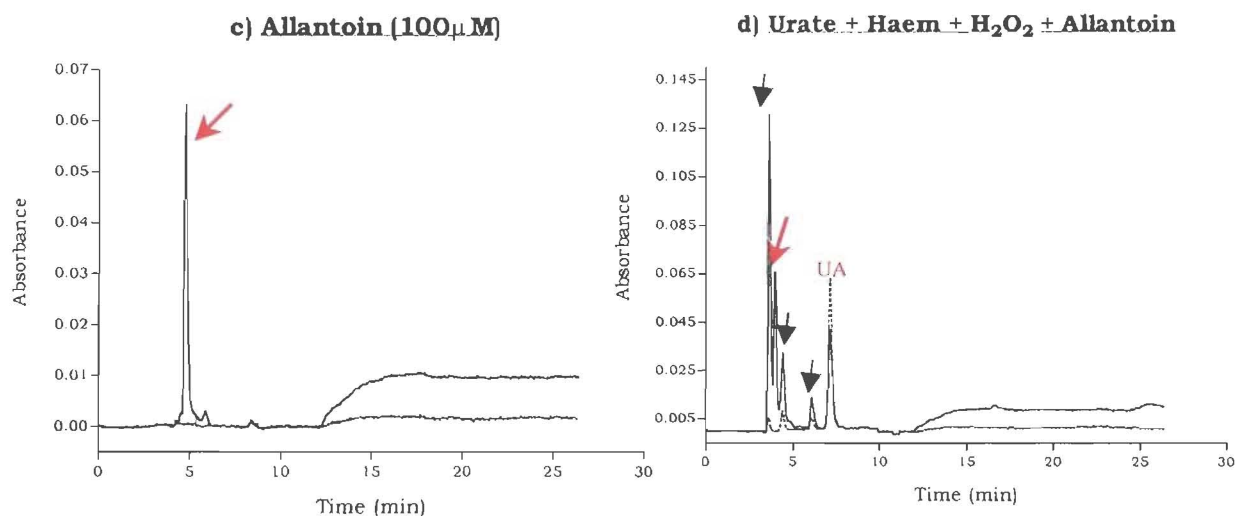

FIGURE 14: HPLC profiles showing the spiked reaction. **c:** allantoin (100µM), **d:** uric acid, haem, H_2O_2, and allantoin. In graph **d**, the allantoin marker is indicated by a red arrow, and urate products by arrow heads.
.............: Absorbance read at 280nm
———: Absorbance read at 220nm

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In summary, the above experiments have shown the following:

1. **urate + haem** = no change
2. **urate + H_2O_2** = no change
3. **urate + haem + H_2O_2** = rapid conversion of urate to several products

Allantoin is the well-known urate degradation product found *in vivo* in many mammals. It is produced in the Purine metabolism pathway during a reaction catalysed by uric acid oxidase (or uricase).

For this study, reaction mixtures were set up (as shown in table 7) to compare the uricase reaction to the "haem catalysed reaction".

Table 7: Experimental setup for the uricase reaction

Mixture	Urate (25µM)	Uricase (2µg/ml)	Allantoin (100µM)
e	+	+	-
f	+	+	+

The experiments were performed in a similar fashion, with reactants being mixed together then allowed to react for 40 seconds before HPLC analysis. Here too allantoin was used as a marker to confirm the identity of the reaction products.

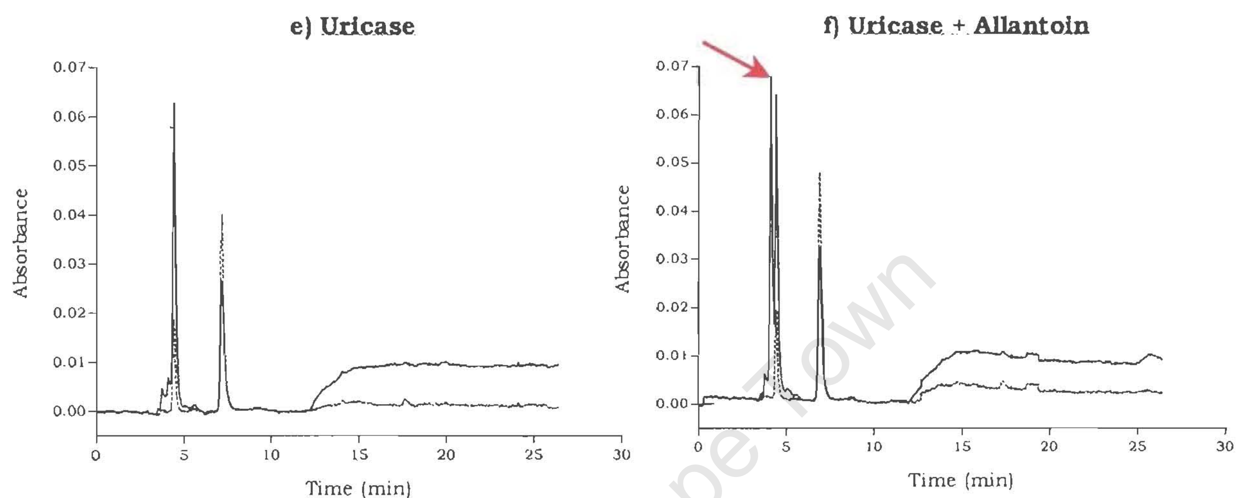

FIGURE 15: Uricase reaction. **e:** uricase (2μg/ml), and uric acid (25μM). **f:** allantoin (100μl), uricase (2μg/ml), and uric acid (25μM).
.............: Absorbance read at 280nm
―――――: Absorbance read at 220nm

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In the same way as with the haem reaction, the uricase reaction occurred rapidly, generating a product (first peak in figure 15e), which for now will be referred to as "the uricase-urate product". Interestingly, when allantoin was added to this reaction as a marker, the uricase-urate product and the allantoin-marker did not elute at the same time. The marker (indicated by an arrow in figure 15f) eluted slightly earlier than the "uricase-urate product", which came as a surprise since the "uricase-urate product" was expected to elute at the same position as the marker. This observation may suggest that the allantoin produced in the uricase reaction, does not have a structure identical to that of the allantoin marker used to spike the uricase reaction. It then follows that the third peak in figure 14b, which seems to have the same

elution characteristics as the "uricase-urate product", could be allantoin. This would imply that haem, on its own, is able to catalyse the degradation of urate, in the presence of H_2O_2, in a similar fashion to uricase by producing allantoin.

3.5.2 Catabolic effects of Haem and H_2O_2 on xanthine and hypoxanthine:

The catalytic abilities of haem were tested on Xanthine (Xa) and Hypoxanthine (Hx), which are precursors to uric acid in the Purine catabolic pathway, using the same protocol as the one applied in the previous section. However, the addition of haem, with or without H_2O_2, to these two compounds, produced no observable effects, i.e. the Hx- and Xa-levels remained unchanged in test reactions (data not shown).

3.5.3 Effects of other oxidants:

Similar experiments, as above, were performed using Hypochlorous acid (HOCl) and AAPH instead of H_2O_2 in order to observe and compare their catalytic effects on uric acid. This set of experiments also produced different results:

- **HOCl** rapidly degraded urate in the presence and absence of haem. Urate products, generated during this reaction, could not be identified because the HPLC profiles showed no identifiable peaks (data not shown).
- **AAPH:** Results of the *in* vitro experiments with AAPH did not show any effect of the peroxyl radical generator on urate (in the presence or absence of haem); As figure 16 shows, the urate peak was unaffected and no new identifiable species were generated. This did not confirm

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the results obtained in figure 8 (urate and tyrosine ORAC assay) which clearly indicated that urate could scavenge peroxyl radicals generated by AAPH, in a reaction that modified urate. It is unclear why the ORAC results did not agree with these experiments.

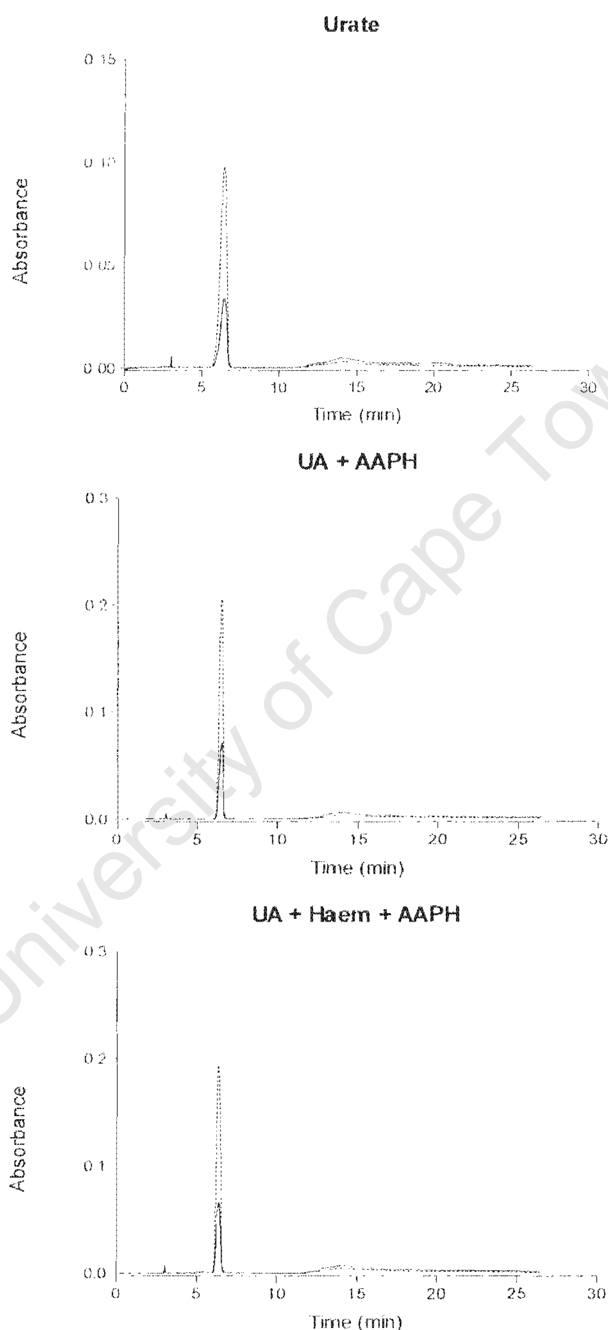

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FIGURE 16: *In vitro* reactions of AAPH on urate in the presence and absence of haem
............: Absorbance read at 280nm
———: Absorbance read at 220nm

3.6 Urate and Tyrosine transport across erythrocyte membranes:

Various studies have shown that urate and tyrosine are able to diffuse across human erythrocyte membranes [18, 19, 23]. These observations were made without the use of HPLC.

In this study, urate and tyrosine transport was investigated by monitoring urate- and tyrosine-peak area variations in erythrocyte extract using reverse-phase HPLC. This investigation was performed on both human and horse erythrocytes, in an attempt to link membrane transport to the erythrocyte urate levels variations observed in race horse extracts in section 3.1.

Separated horse and human erythrocytes were incubated at a 20% hematocrit for 30min in a medium containing either uric acid (0.5mM) or tyrosine (1mM). The cells were then washed, and some were prepared for HPLC analysis (section 2.4) so that any uptake of urate or tyrosine could be observed. The rest of the cells were further incubated in Hanks buffered solution for 2hrs then also analysed on HPLC to see whether the tyrosine or the urate that might have entered the cells during the first incubation had been retransported across the erythrocytes membranes back into the medium.

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3.6.1 Tyrosine transport:

During the tyrosine-transport experiments, erythrocytes were incubated for 30min in a buffer containing 140mM NaCl, 5mM KCl, 2mM $MgCl_2$, and 15mM Tris [19]. Tyrosine was added to the buffer to a final concentration of 1mM.

Figure 17, below, shows HPLC profiles of horse erythrocytes.

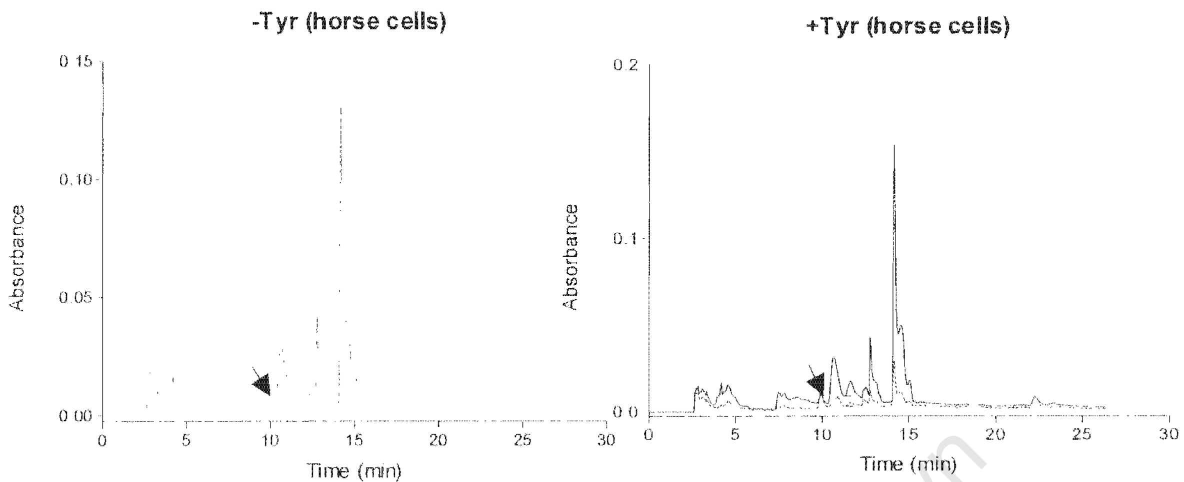

FIGURE 17: Tyrosine transport experiments on horse red blood cells: **-Tyr:** control, **+Tyr:** red cell extracts incubated in the presence of 1mM tyrosine (Tyrosine is indicated by an arrow).
.............: Absorbance read at 280nm
————: Absorbance read at 220nm

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As the HPLC profiles show, erythrocyte incubation in the presence of tyrosine did not result in any change within the erythrocytes. This indicates that there was no uptake of tyrosine by the horse erythrocytes.

Experiments with human erythrocytes, however, produced different results as shown in figure 18 below.

The species eluting at 6min in figures 18a, 18e and 18f (indicated by blue arrows) are consistent with the eluting position and OD 280/260 ratio of uric acid.

Control cells show no tyrosine and very little uric acid (fig. 18a). However, tyrosine (indicated by a red arrow in fig. 18b) was readily taken up by the erythrocytes after a 30min-incubation in the presence of 1mM tyrosine. Cells that were incubated for a further 2hrs in Hanks buffered solution were extracted then analysed on HPLC, and so was the Hanks solution used to

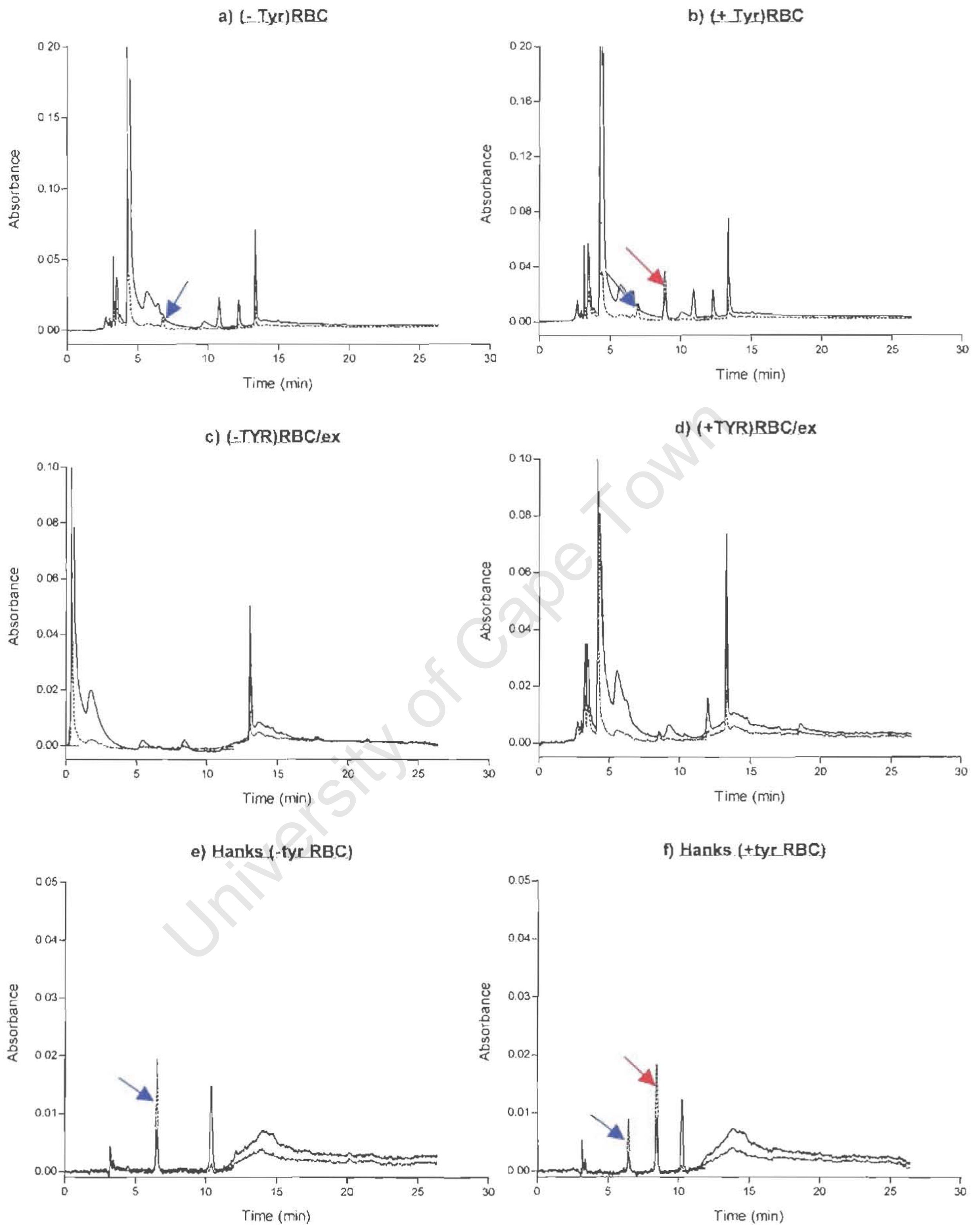

FIGURE 18: Tyrosine uptake experiments. **a:** Control, **b:** Tyrosine loaded, **c:** Control with continued incubation in Hanks solution for 1hr, **d:** Tyrosine loaded cells incubated for 2hrs in Hanks, **e:** Hanks solution used to incubate *c*, **f:** Hanks solution used to incubate *d*. Tyrosine is indicated with a red arrow, while the blue arrows indicate urate peaks.

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incubate the cells. Figure 18d shows that erythrocytes lost the tyrosine that had been taken up during the first incubation, and, as figure 18f shows, this tyrosine was re-transported into the medium. This observation rules out the possibility that tyrosine was metabolised within the erythrocytes.

These experiments are not quantitative but simply demonstrate the presence of tyrosine transport in human erythrocytes.

3.6.2 Urate transport:

Erythrocyte membranes contain a band-3 protein consisting of a dimer with a membrane spanning domain which is responsible for anion transport. Unlike many other transporters, the band-3 protein is a non-selective transporter as it is able to transports various anions across the membrane [26]. Since it was shown that urate enters erythrocytes in the anionic form [18], it can be suggested that band-3 is likely to play a major role in the transport of urate across erythrocyte membranes.

Urate transport experiments were initially performed here with PBS-glucose and "tyrosine-uptake" buffers [28, 19], both of which had a strong anionic content (Materials and Methods, section 2.7). However, no accumulation of urate was ever observed in the erythrocytes when using these buffers. If urate transport occurs through the band-3 anion transporter, then, during the initial uptake experiments, urate could have been competing with other anions present in the buffers (i.e. phosphate ions, chloride ions ...) for entry into the erythrocytes, resulting in a difficulty to observe urate accumulation in the erythrocytes. To avoid this competition, a medium which contained no anions apart from urate was needed. A Sucrose medium, containing 260mM Sucrose and 20mM hepes (Materials and Methods, section 2.7), fulfilled this

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requirement. This medium had been succesfully used by D. Black in his study on erythrocyte transport of the anion orotate [21].

The protocol for the urate uptake experiments was otherwise similar to that used for the tyrosine uptake experiments, and the amount of urate used in the incubation medium was 0.5mM.

The experiments were once again unsuccessful with horse erythrocytes, showing no uptake of urate by the cells (figure 19).

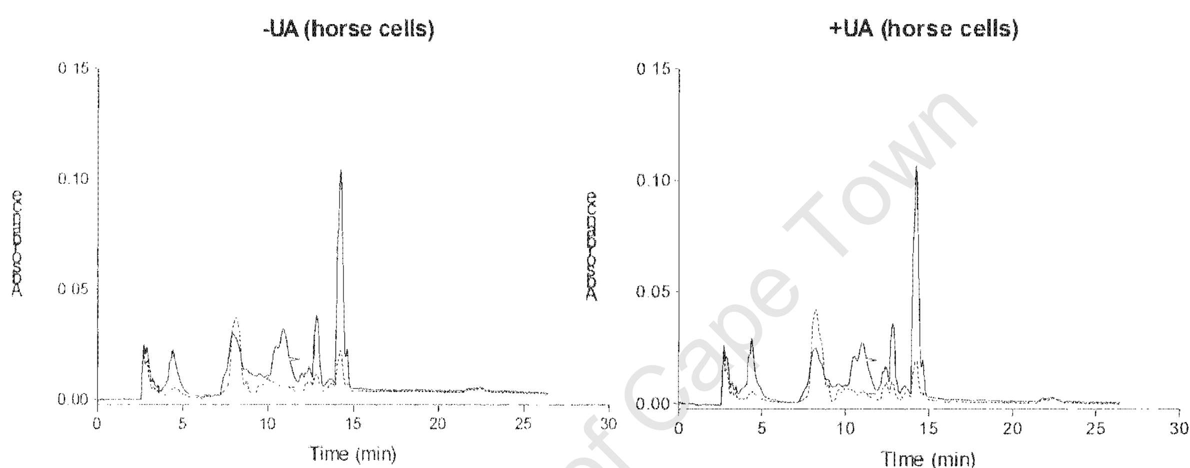

FIGURE 19: Uric acid transport experiments on horse red blood cells. *The curve anomaly at 12-min is an artefact of the HPLC column.*
............: Absorbance read at 280nm
———: Absorbance read at 220nm

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HPLC analyses for human erythrocytes gave the results shown in figure 20 below. These showed that a transport system, which is detectable in the cells after less than 30min of incubation in "sucrose-buffer" (figure 20b), also operates for urate. Urate concentration in figure 20b extract was approximately 42μM. Taking into consideration the erythrocyte extracts were diluted 1:4 with saline and PCA during HPLC-preparation (section 2.4), it can be deduced that the original amount of urate in the whole erythrocytes was about 170μM.

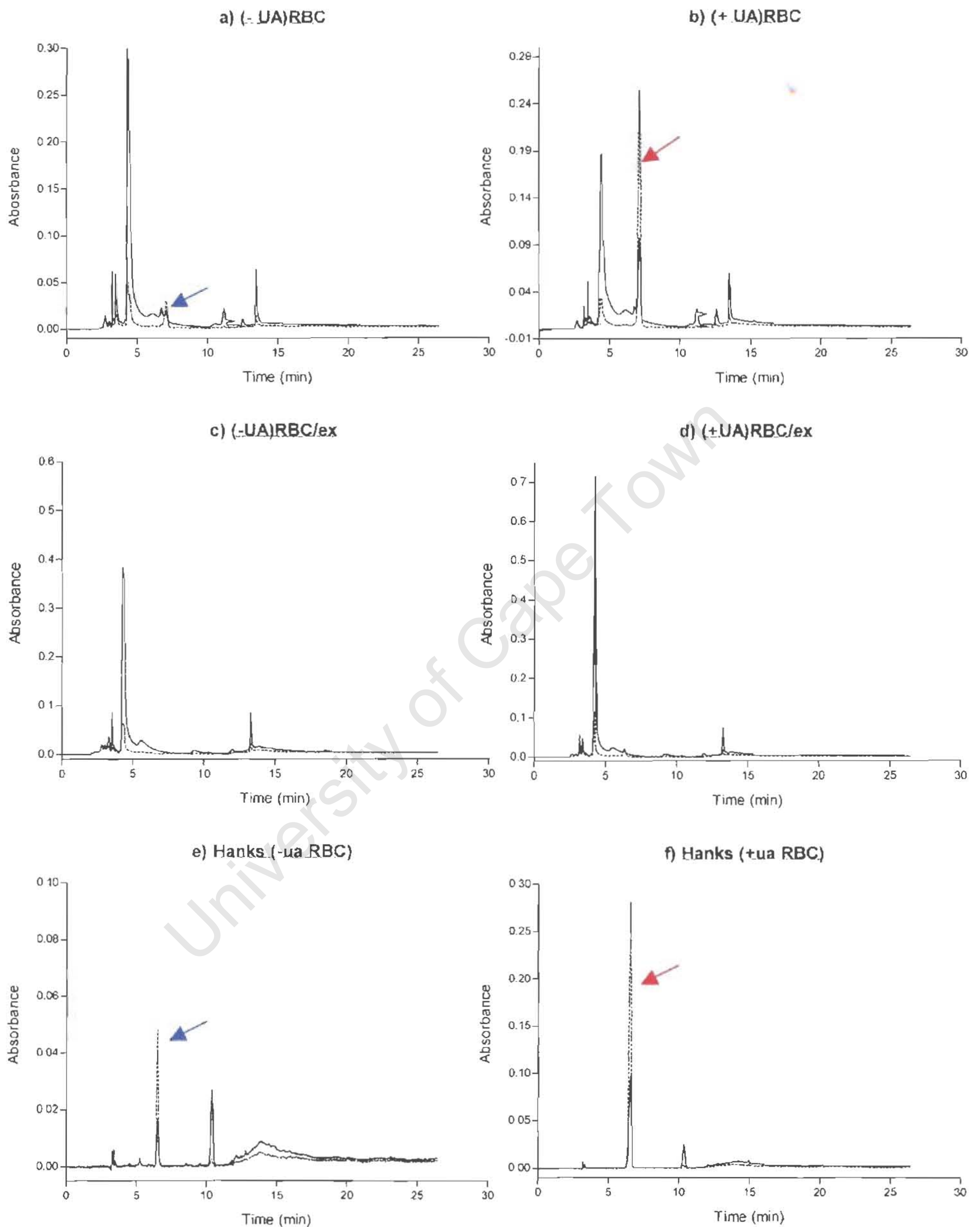

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FIGURE 20: Urate uptake experiments. **a:** Control, **b:** Urate loaded *The curve anomaly at 12-min, in these two figures, is an artefact of the HPLC column.*, **c:** Control with continued incubation in Hanks solution for 1hr, **d:** Urate loaded cells incubated for 2hrs in Hanks, **e:** Hanks solution used to incubate *c*, **f:** Hanks solution used to incubate *d*. Urate that was taken up is indicated with a red arrow, whereas the urate that was already present in the cells is indicate with a blue arrows.

Urate also readily exited the cells when the erythrocytes were re-incubated in Hanks solution containing no added uric acid. After 2hrs of incubation, the erythrocytes contained virtually no urate (figure 20d), while Hanks solution contained 45μM of urate (approximately 180μM present in the original erythrocytes) (figure 20f), which indicates that urate was not broken down within the cells, but rather re-transported into the medium, as was the case for tyrosine.

In summary, urate and tyrosine uptake has successfully been demonstrated in human erythrocytes. The transport systems responsible for these uptakes may be linked to antioxidant mechanisms, allowing tyrosine and urate to enter the erythrocytes' intracellular environment to scavenge free radicals accumulated during oxidative stress.
Horse erythrocytes did not show any uptake of either urate or tyrosine. However, the observed presence of urate and tyrosine in horse erythrocytes, may suggest that these substances are either produced within the horse erythrocytes themselves by some unknown metabolic process, or that tyrosine and urate precursors are transported into erythrocytes, then converted into tyrosine or urate. For example tyrosine might cross erythrocyte membranes as tyrosine chloramine, and urate as hypoxanthine.

3.7 Tissue culture experiments:

Having established that urate and tyrosine can be transported across human erythrocyte membranes, attempts were made to understand in what way these two compounds take part in antioxidant mechanisms within erythrocytes.

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Erythrocytes have been shown to protect neutrophils and T-lymphocytes, against apoptosis induced by various factors, such as oxidants [16, 17]. Experiments were therefore conducted in this study to see whether erythrocytes could also protect cultured fibroblasts against free radicals.

Fibroblast cells were labelled with the radioactive substrates ^{14}C-uridine and ^{3}H-phenylalanine, which were easily incorporated by the cells into RNA and protein respectively. Substrate uptake was simply monitored by measuring incorporation of label into acid-precipitable material using a Liquid Scintillation Counter (1600 TR, Packard).
The fibroblasts were put under oxidative stress by exposure to the peroxyl radical generator AAPH. Free radical damage to the cells was monitored by measuring the decrease in radioactive label uptake by the fibroblasts. Both labelled compounds were used together to assess whether RNA synthesis or protein synthesis was the most efficient way to monitor cell damage.

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3.7.1 Experiment 1:

The first step was to investigate the antioxidant capacity of human erythrocytes was by adding 1μl and 10μl of a solution of diluted erythrocytes (1:100 dilution in Hanks buffered solution) to fibroblast cells plated onto 24 well-plates, in the absence (1-3 in table 8) or presence (4-9 in table 8) of the peroxyl radical generator AAPH. Table 8 below shows the set up of the experiment, with each cell of the table indicating the set up of each experimental well (each of these wells was duplicated).

Table 8: Experiment 1set up. Each experiment was performed in duplicate.

1	2	3
No AAPH No RBC	No AAPH 1μl RBC	No AAPH 10μl RBC
4	**5**	**6**
40mM AAPH No RBC	40mM AAPH 1μl RBC	40mM AAPH 10μl RBC
7	**8**	**9**
80mM AAPH No RBC	80mM AAPH 1μl RBC	80mM AAPH 10μl RBC

After a 4hr-exposure to 1μCi ^{3}H-Phenylalanine and 0.1μCi ^{14}C-Uridine, label-incorporation was measured with a Liquid Scintillation Counter, and label counts obtained from this analysis were plotted as histograms (see figure 21. The data obtained is given in Table A of the Appendix).

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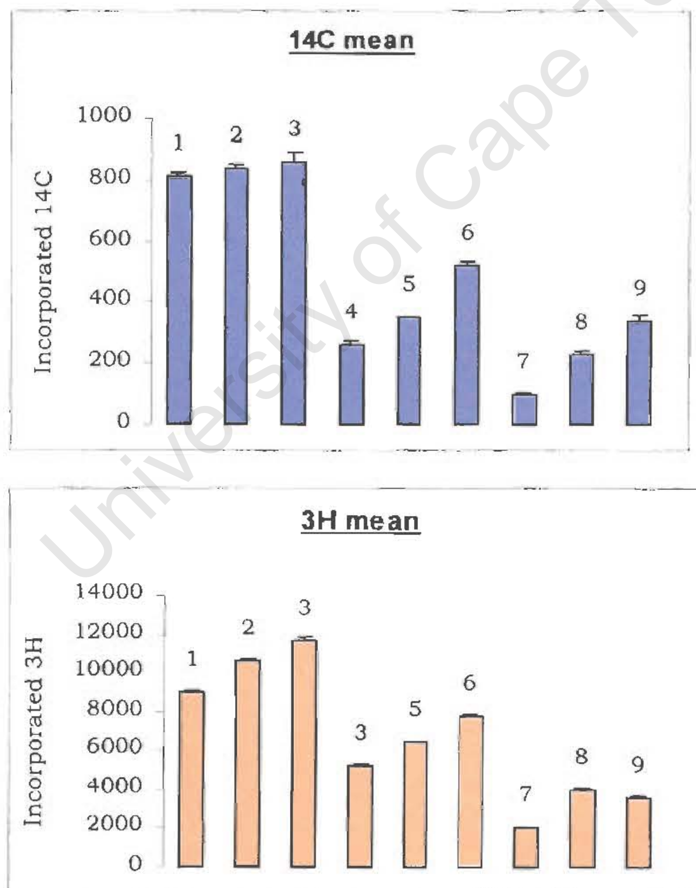

FIGURE 21: Experiment 1. ^{14}C-Uridine (0.2μCi) and ^{3}H-Phenylalanine (1μCi) label counts on fibroblast cells exposed to **(1-3):** no AAPH, **(4-6):** 40mM AAPH, **(7-9):** 80mM AAPH. The number denoting each bar corresponds to an experiment shown in Table 8 with the same number.

In the series of "control"-experiments (1-3), which were conducted in the absence of any oxidant, there was little variation in uptake of ^{14}C-uridine by fibroblasts when adding different amounts of erythrocyte, however, ^{3}H-phenylalanine uptake showed more variation. A reason for this may be that small amounts of erythrocytes enhanced protein synthesis in fibroblasts due to oxygen delivery or through some other means.

The addition of AAPH to the fibroblast cultures produced clear cell damage as indicated by the decrease in label uptake (4-9), however, the addition of 1μl and 10μl of erythrocyte-solution caused some level of protection against oxidative damage when added to the experiment.

This experiment hence showed some evidence of protection of fibroblasts by erythrocytes in the presence of AAPH.

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3.7.2 Experiment 2:

In the second experiment, label uptake by fibroblasts was monitored using different substrates, and labels were interchanged between nucleotide and amino acid: ^{3}H-Phenylalanine to ^{3}H-Thymidine and ^{14}C-Uridine to ^{14}C-Iso-Leucine. This was done to see whether similar uptake patterns for each label could be obtained in spite of a change in substrate type, to ensure that label-uptake did not depend on the type of substrate taken up by the cells (i.e. nucleotide or amino acid). The experimental set up was as described in table 9.

Table 9: Experiment 2 set up. The experiments were performed in quadruplicate.

1	2	3
No AAPH No RBC	No AAPH 1μl RBC	No AAPH 10μl RBC
4	**5**	**6**
80mM AAPH No RBC	80mM AAPH 1μl RBC	80mM AAPH 10μl RBC

Labels were added at the same activities (1μCi ^{3}H-Thymidine and 0.2μCi ^{14}C-IsoLeucine), and each experiment was performed in quadruplicate.

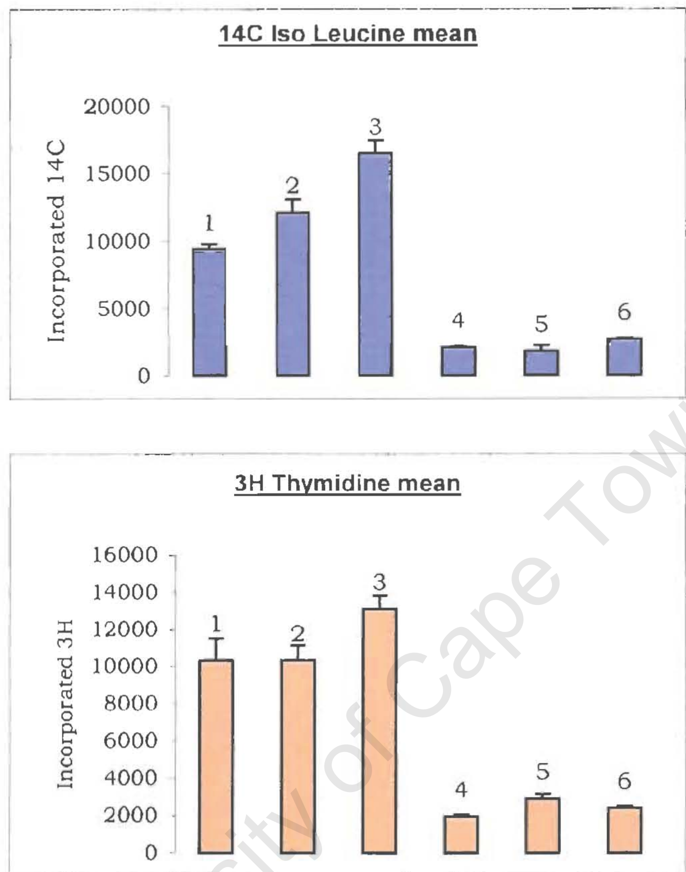

FIGURE 22: Experiment 2. ^{14}C-IsoLeucine (0.2μCi) and ^{3}H-Thymidine (1μCi) label counts on fibroblast cells exposed to **(1-3):** no AAPH, **(4-6):** 80mM AAPH.

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Generally, the labelling patterns that were obtained (as seen in figure 22.The data obtained is given in Table B in of the Appendix)) showed more variation for ^{14}C-IsoLeucine, indicating, once again, that erythrocytes apparently enhanced uptake of protein by fibroblasts. ^{3}H-Thymidine nucleotide uptake, on the other hand, remained more or less constant. erythrocyte protection was, however, less convincing in this experiment.

It can be concluded that the uptake of label by the fibroblast cells is generally dependent on the type of substrates that the cells are exposed to.

3.7.3 Experiment 3:

Different mammalian erythrocytes were tested, in a third experiment, in order to see whether the ability of erythrocytes to protect against oxidative stress was species-specific.

The erythrocytes used here were obtained from Human (*H*), Black Rhinoceros (*Rh* - which was found to contain large amounts of tyrosine [22]), Horse 1 (*h1* - which, as observed by HPLC, contained urate – data not shown), and Horse 2 (*h2* – which contained no urate – data not shown).

As table 10 shows, 1μl and 10μl, of each erythrocyte solution, was added to fibroblasts in the presence or absence of AAPH.

Table 10: Experiment 3 set up. These experiments were done in quadruplicate. *h1*: urate-containing horse erythrocytes, *h2*: horse erythrocytes with no urate, *H*: human erythrocytes, *Rh*: black rhinoceros erythrocytes.

No AAPH	**1** No RBC	**2** 1μl *H*	**3** 10μl *H*	**4** 1μl *h1*	**5** 10μl *h1*
	6 1μl *h2*	**7** 10μl *h2*	**8** 1μl *Rh*	**9** 10μl *Rh*	
100mM AAPH	**10** No RBC	**11** 1μl *H*	**12** 10μl *H*	**13** 1μl *h1*	**14** 10μl *h1*
	15 1μl *h2*	**16** 10μl *h2*	**17** 1μl *Rh*	**18** 10μl *Rh*	

Here too experiments were performed in quadruplicate. Figure 23, shows the results (the data obtained is given in Table C of the Appendix).

As observed in Experiment 1, uptake of label was generally increased in the presence of the erythrocytes. This occurred with human and horse erythrocytes, but not with rhinoceros cells, where label uptake remained constant.

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The experiments performed in the presence of AAPH did not clearly indicate whether the efficiency in protecting against oxidative damage is species-specific. The presence of urate and tyrosine in sample-pairs **13,14** and **17,18**, respectively, did not cause significant changes that could have

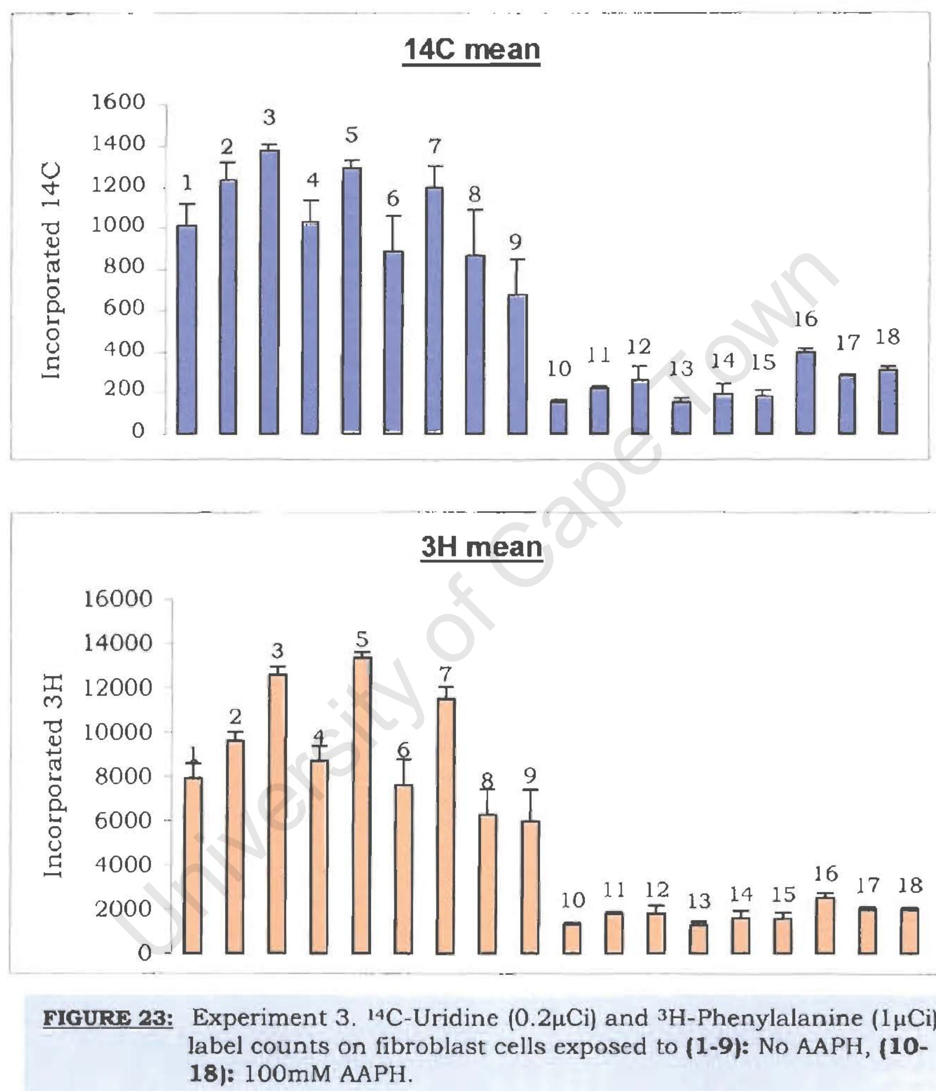

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FIGURE 23: Experiment 3. ^{14}C-Uridine (0.2μCi) and ^{3}H-Phenylalanine (1μCi) label counts on fibroblast cells exposed to **(1-9):** No AAPH, **(10-18):** 100mM AAPH.

indicated more protection. Sample 16 showed higher uptake than the other samples, but the reasons for this are unclear.

The protocol used for these experiments could be improved using a double label approach in order to reduce well-to-well cell number variation. Other

oxidants could also be tested using the same experimental set up in order to compare their effect to that of AAPH.

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Chapter 4: DISCUSSION and CONCLUSIONS

Various researches have shown that blood purine levels, in particular urate levels, increase in human blood plasma with exercise [13, 29, 30, 31]. This study has shown that similar mechanisms occur within erythrocytes. Initially, horse samples were analysed by HPLC, where urate and tyrosine were positively identified as significantly identifiable species in the HPLC profiles. Tyrosine has been observed previously as a major component of erythrocytes from species in the mammalian order *Perissodactyla* (horses and rhinoceroses) [22]. The presence of large quantities of urate within erythrocytes came as a surprise as it is generally assumed that erythrocytes lack the enzymatic machinery to produce urate. A component of this project was thus devoted to trying to understand urate metabolism within erythrocytes, and to observe the fluctuations of the levels of urate in erythrocytes before and after exercise.

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Horse erythrocyte HPLC profiles showed unexpected dynamics of urate during and after exercise, showing major degrees of urate level variation over time. The urate fluctuations were unique to each horse making it difficult to draw up a clear variation pattern, but a longer-term analysis showed, with statistical significance, that exercise contributed to an overall increase of urate levels in erythrocytes.

Similar exercise experiments were conducted on a human subject, but the results did not show any effect on uric acid levels in erythrocytes.

Studies have shown that levels of other purines vary in plasma during and after exercise. For example, 10 min after termination of exercise in man, the production of hypoxanthine is 3 times higher than at rest [30].

Ames et al. [13] proposed that a variation in the levels of uric acid in plasma was indicative of a physiological mechanism that helps the body cope with oxidative stress. Urate is a known powerful soluble anti-oxidant [11,12,27] and this variation might indicate a process of recovery where more urate is produced to scavenge free radicals generated during and after exercise.

When they carry oxygen, erythrocytes are more susceptible to oxidative stress in the event of reduction mechanisms that can easily occur on the oxygen molecule. Many *in vitro* and *in vivo* studies have shown that several parameters of erythrocyte function and integrity are negatively affected by increased oxidative stress [13,29]. When challenged with various oxygen radical generating systems, erythrocytes show one or several of the following reactions: changes of erythrocyte membrane ionic permeability, increase of lipid peroxidation, oxidation of protein sulfhydryl groups and activation of proteolysis [29]. Erythrocytes may be even more susceptible to oxidative stress during exercise because of the greater oxygen flux through haemoglobin the blood at that time. Three percent of human oxygenated haemoglobin normally undergoes autoxidation to yield methaemoglobin with the concomitant production of superoxide. This superoxide is then converted to H_2O_2 and O_2, and the H_2O_2, if not destroyed by catalase or glutathione peroxidase, can react with the iron in the haemoglobin to form oxo-haem oxidants. These oxidants are important initiators of lipid peroxidation in erythrocyte membranes [13].

Erythrocytes have, however, only a certain level of tolerance to oxidative stress beyond which erythrocyte membrane modifications irreversibly alter

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the structure and function of the membrane, allowing the escape of macromolecules, and haemolysis [29]. Hence the necessity, in erythrocytes, for the presence of strong antioxidant mechanisms which include the antioxidant enzymes catalase, superoxide dismutase, and glutathione peroxidase which efficiently scavenge reactive oxygen species (ROS).
With an ORAC assay it was shown that urate and tyrosine have similar antioxidant capacities, and are able to scavenge peroxyl radicals. The ORAC values obtained for each of these compounds were 7.0μmol/l and 8.7μmol/l respectively. Further ORAC analyses showed that the antioxidant abilities of urate are preserved within horse erythrocyte extracts. This may imply that urate and tyrosine form part of non-enzymatic antioxidant systems within the erythrocytes, which means that they would be able to provide a first line of anti-oxidant defense by scavenging free radicals produced during exercise within the erythrocytes.

Uric acid levels in erythrocyte extracts were observed to vary markedly over short time intervals. An investigation into the cause of uric acid disappearance from erythrocytes showed that storage conditions markedly influence urate levels within human erythrocytes or in erythrocytes extracts. The reason for the disappearance of urate from intact erythrocytes could be that upon exposure of these erythrocytes to extracellular oxygen during the experiments, the production of ROS within the cells is induced, stimulating scavenging by catalases, dismutases, and probably also urate.
During PCA extractions , all proteins, including antioxidant enzymes, are precipitated resulting in erythrocyte extracts that only contain small molecules including urate and haem. It is in these conditions that this urate was found to degrade rapidly while scavenging ROS.

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Interest was directed towards haem as a potential key agent in the degradation of uric acid in erythrocytes and erythrocyte extracts, as it has been shown, in various studies, to possess catalytic abilities. Howell and Wyngaarden [24] showed the catalytic abilities of hemoglobin in the presence of a peroxide, and concluded that haemoglobin catalyzed the degradation of urate when it was in the methaemoglobin (metHb) form. In their study, hematin (or haem) itself was also found to be an excellent catalyst for urate peroxidation mainly because it formed well defined complexes with H_2O_2.

A number of other hemoproteins are also known to catalyze the peroxidation of urate. These include myeloperoxidase, horseradish peroxidase, lactoperoxidase, verdoperoxidase and cytochrome c [24]. The component common to these proteins and to MetHb is the trivalent iron porphyrin prosthetic group. However, the pH optima of these proteins are all quite different from one another [24].

The present study demonstrated the catalytic abilities of haem *in vitro* through HPLC analyses, and indicated that the degradation of uric acid was followed by the production of at least 3 products in the presence of hydrogen peroxide. One of these products shared similar characteristics to allantoin, evidenced by HPLC analyses of the products of a uricase catalysed reaction on urate (section 3.5.1).

The allantoin-like product produced in these reactions appeared unstable, and hence could not be adequately processed for Mass Spectrophotometry analysis, which would have helped confirm the identity of the product. According to Howell and Wyngaarden [24], the nature of the products obtained from the oxidation of urate depends upon the buffer employed and the pH to which the reaction mixture is subsequently adjusted. They identified allantoin as a product of oxidation of urate by hematin and

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peroxide at pH 7.5. However, they could recover only part of uric acid as allantoin, suggesting possible further oxidation products [24]. It was also shown that the products of oxidation of uric acid catalysed by uricase or by peroxidase are identical when brought to the same pH.

One could then suggest that, in the context of the experiments performed during this study, haem was one of the main elements responsible for the disappearance of urate from erythrocytes and erythrocyte extracts, in a reaction dependent upon the presence of a peroxide.

This study was not able to demonstrate whether haem could catalyze the degradation of other purines. The urate precursors, hypoxanthine and xanthine, did not seem to be affected when they were exposed to hydrogen peroxide and haem. This suggests that catalysis by haem seems to be restricted to uric acid itself. The effect of haemoglobin itself has, however, not yet been tried on hypoxanthime and xanthine.

Other oxidants did not produce the same effects on urate as hydrogen peroxide. HOCl degraded urate but with no observable products on HPLC, and AAPH showed no observable effect on urate when analyzing the products on HPLC. This last observation seems, however, to contradict results obtained during the ORAC assays, where it seemed clear that there was an interaction between urate and peroxyl radicals generated by AAPH. It is as yet unclear why there is a conflict between the ORAC results and the in vitro AAPH results, and the experiments need to be repeated.

Transport studies on horse erythrocytes did not show any evidence of urate or tyrosine transport across erythrocyte membranes *in vitro*. However, the transport of these two compounds could clearly be shown in human cells.

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It is unclear, however, what the mechanism of production of uric acid is in the erythrocyte although it appeared likely to be associated with increased oxidative stress. Urate is normally produced in the liver, but only in man and in the higher apes does it accumulate in plasma since most other mammals have uricase in the liver, degrading urate to allantoin. The urate, which is observed in erythrocytes, could play a role in protecting the erythrocytes against oxidant-induced hemolysis, but it is also possible that it reflects purine degradation and/or anti-oxidant activity in peripheral tissues such as exercising muscles, with purines such as hypoxanthine taken up from these sites by the erythrocyte and converted by some as yet not identified oxidating process to urate. Haem or haemoglobin could then, in the presence of further oxidants from oxyhaemoglobin or from peripheral tissues again, be responsible for the further metabolism of uric acid once the latter is in the erythrocytes.

As mentioned earlier, other purines are also metabolized during oxidative stress. As soon as oxidative stress starts to deplete ATP, more AMP is made available to AMP-deaminase, the activity of which is dramatically increased by oxidative stress [29]. AMP is, therefore, rapidly converted to IMP, which in turn increases up to 20-fold. Excess IMP is partly accumulated inside the erythrocytes and, in man, partly lost into the plasma as inosine (Ino), hypoxanthine (Hx), xanthine (Xa) and urate, all of which are able to permeate freely through the erythrocyte membrane [29].

Increased levels of Hx in plasma during exercise were confirmed in a study performed by Sahlin et al. [30], where it was also demonstrated that the source of the progressive increase of Hypoxanthine, during exercise and recovery, is the working muscle [30].

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Hypoxanthine originating from the working muscle could hence either diffuse into the plasma and then enter the erythrocyte to form urate (which would either require the presence of xanthine oxidase (XO) within the erythrocytes, or some other unidentified non-enzymatic process), or urate would be formed within the muscle cell, diffuse into the plasma and then enter the erythrocytes. The second option is more plausible, as the presence of XO in erythrocytes has never been demonstrated. However, for horse erythrocytes, which do not possess a transporter for urate, there could be another mechanism facilitating the accumulation of urate within the erythrocytes. The uptake and metabolism of Hx by horse erythrocytes needs to be studied in more detail in order to investigate this possibility.
Schematically, the mechanism governing urate metabolism in erythrocytes could be as shown in figure 24.
As discussed in the Introduction, erythrocytes have the ability to protect other cell types, namely, T-cells and Neutrophils. The tissue culture experiments, in this project, have shown that erythrocytes, from various mammalian species are able to also protect fibroblast cultures against oxidants. This could be due to the presence of strong antioxidant enzymes within erythrocytes as well as soluble anti-oxidants. However, the presence of urate or tyrosine within the erythrocytes could not be demonstrated to significantly increase the protective effect of erythrocytes.

In Conclusion, the antioxidant roles of uric acid and tyrosine have been discussed with an emphasis on urate metabolism. Although the specific steps of this process are still unclear, it is possible that urate is associated with mechanisms maintaining the redox homeostasis of the body. The possibility that urate might play a protective role for erythrocytes, and other

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tissues, is an intriguing possibility. However, further studies need to be done in order to uncover the exact mechanism through which uric acid is able to achieve this role.

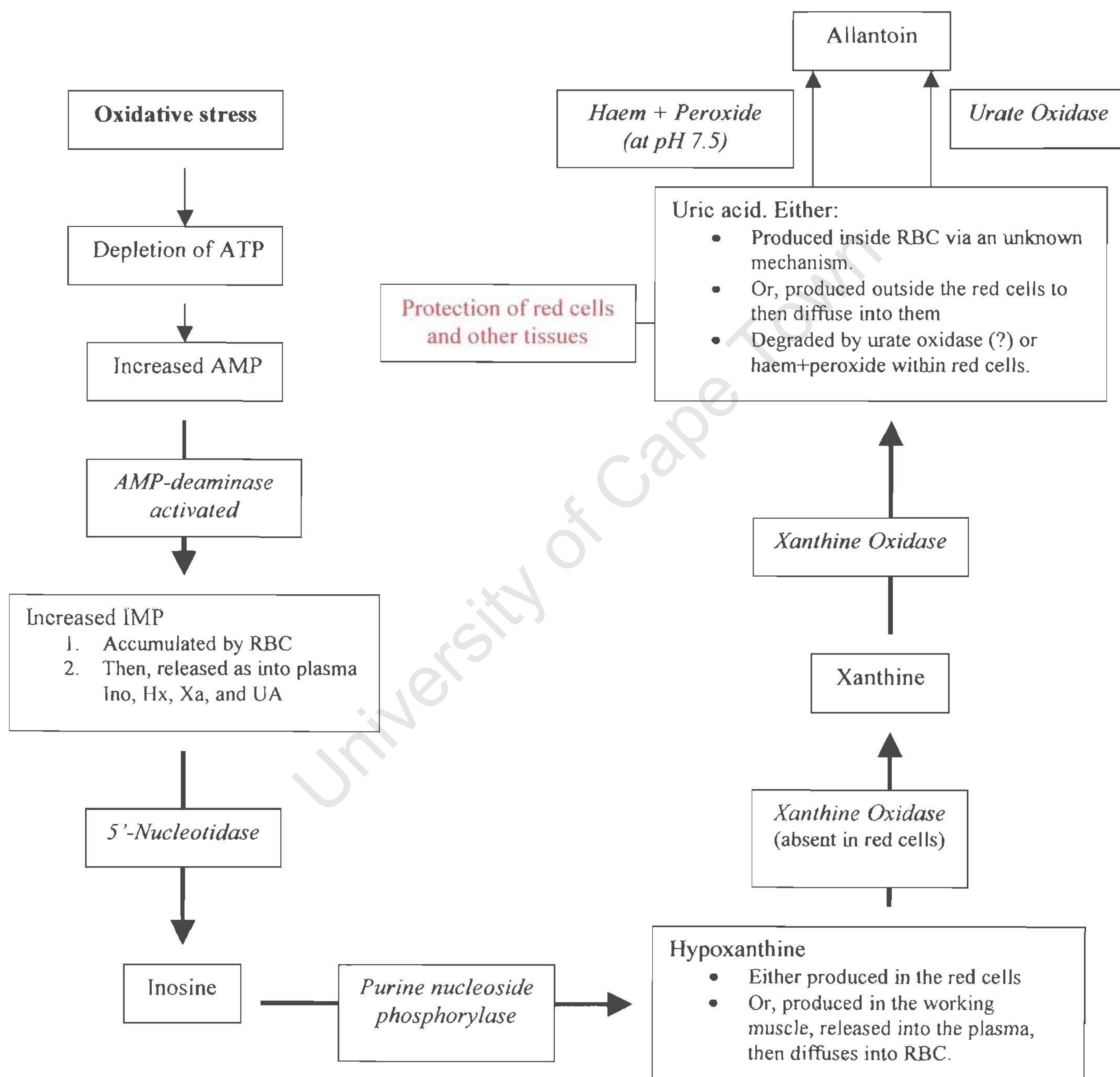

FIGURE 24: Scheme of possible mechanisms governing urate diffusion into erythrocytes.

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APPENDIX

Table A: Experiment 1: ^{14}C-Uridine (0.2μCi) and ^{3}H-Phenylalanine (1μCi) label counts on fibroblast cells.

RBC (μl)	AAPH (mM)	3H	3H mean	STD dev	STD err	14C	14C mean	STD dev	STD err
0	0	9152.0	9000.0	146.4	103.5	818.0	810.0	21.2	15
0	0	8945.0				788.0			
1	0	10548.0	10600.0	113.1	80	841.0	840.0	13.4	9.5
1	0	10708.0				822.0			
10	0	11415.0	11650.0	359.9	254.5	825.0	860.0	44.5	31.5
10	0	11924.0				888.0			
0	40	5078.0	5200.0	120.2	85	269.0	260.0	15.5	11
0	40	5248.0				247.0			
1	40	6421.0	6450.0	41.7	29.5	340.0	350.0	4.2	3
1	40	6480.0				346.0			
10	40	7829.0	7750.0	123.0	87	524.0	520.0	14.1	10
10	40	7655.0				504.0			
0	80	1937.0	2000.0	16.9	12	94.0	100.0	1.4	1
0	80	1961.0				96.0			
1	80	3875.0	4000.0	165.5	117	212.0	230.0	17.7	12.5
1	80	4109.0				237.0			
10	80	3531.0	3600.0	94.0	66.5	319.0	340.0	24.7	17.5
10	80	3664.0				354.0			

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Table B: Experiment 2: ^{14}C-IsoLeucine (0.2μCi) and ^{3}H-Thymidine (1μCi) label counts on fibroblast cells.

RBC (μl)	AAPH (mM)	3H	3H mean	STD dev	STD err	14C	14C mean	STD dev	STD err
0	0	12635.0	10300.0	2413.9	1207.0	9586.0	9390.0	794.3	397.1
0	0	13801.0				10325.0			
0	0	8638.0				8416.0			
0	0	9753.0				9213.0			
1	0	9063.0	10325.0	1602.9	801.5	9176.0	12110.0	1956.3	978.2
1	0	12301.0				12962.0			
1	0	12342.0				13096.0			
1	0	12151.0				13194.0			
10	0	12969.0	13100.0	1446.4	723.2	14005.0	16480.0	1933.6	966.8
10	0	15891.0				17718.0			
10	0	15914.0				18276.0			
10	0	14077.0				15903.0			
0	80	2231.0	1950.0	190.7	95.3	2289.0	2090.0	224.5	112.2
0	80	2243.0				2244.0			
0	80	1839.0				1803.0			
0	80	2037.0				2016.0			
1	80	2763.0	2900.0	501.0	250.5	2593.0	1820.0	896.4	448.2
1	80	2741.0				2520.0			
1	80	2797.0				752.0			
1	80	3768.0				1393.0			
10	80	2799.0	2400.0	188.1	108.6	2824.0	2700.0	134.0	77.4
10	80	2424.0				2556.0			
10	80	2638.0				2695.0			

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Table C: Experiment 3: ^{14}C-Uridine (0.2μCi) and ^{3}H-Phenylalanine (1μCi) label counts on fibroblast cells.
h1: urate-containing horse erythrocytes,
h2: horse erythrocytes with no urate,
H: human erythrocytes,
Rh: black rhinoceros erythrocytes.

RBC (μl)	AAPH (mM)	3H	3H mean	STD dev	STD err	14C	14C mean	STD dev	STD err
0	0	6615.0	7900.0	1180.4	681.5	800.0	1010.0	181.7	104.9
0	0	8357.0				1048.0			
0	0	8866.0				1154.0			
				H					
1	0	10125.0	9600.0	803.4	401.7	1287.0	1230.0	173.1	86.5
1	0	10144.0				1231.0			
1	0	9880.0				1395.0			
1	0	8461.0				986.0			
10	0	13561.0	12575.0	747.5	373.8	1431.0	1380.0	60.0	30.0
10	0	12501.0				1366.0			
10	0	11745.0				1301.0			
10	0	12729.0				1422.0			
				h1					
1	0	9695.0	8700.0	1341.3	670.6	1210.0	1030.0	208.3	104.1
1	0	7387.0				793.0			
1	0	7851.0				895.0			
1	0	10106.0				1184.0			
10	0	13450.0	13350.0	471.7	235.9	1237.0	1290.0	79.0	39.5
10	0	13095.0				1271.0			
10	0	14102.0				1406.0			
10	0	13108.0				1244.0			
				h2					
1	0	9487.0	7600.0	2338.8	1169.4	1193.0	890.0	335.8	167.9
1	0	5284.0				570.0			
1	0	9790.0				1152.0			
1	0	5959.0				615.0			
10	0	10297.0	11500.0	1042.3	521.1	972.0	1200.0	195.8	97.9
10	0	12849.0				1448.0			
10	0	11527.0				1162.0			
10	0	11598.0				1212.0			
				Rh					
1	0	8513.0	6250.0	2334.1	1167.0	1229.0	870.0	435.7	217.8
1	0	3568.0				349.0			
1	0	7964.0				1230.0			
1	0	5198.0				671.0			
10	0	8869.0	5975.0	2827.1	1413.5	1013.0	670.0	352.7	176.4
10	0	7904.0				901.0			
10	0	3042.0				271.0			
10	0	4164.0				462.0			
0	100	1410.0	1300.0	146.5	73.3	148.0	150.0	28.5	14.3
0	100	1354.0				178.0			

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0	100	1213.0				137.0			
0	100	1084.0				109.0			
				H					
1	100	1795.0	1800.0	169.5	84.8	234.0	220.0	29.3	14.6
1	100	1993.0				246.0			
1	100	1797.0				184.0			
1	100	1578.0				198.0			
10	100	923.0	1800.0	645.2	372.5	116.0	260.0	124.4	71.8
10	100	2038.0				330.0			
10	100	2353.0				333.0			
				h1					
1	100	1372.0	1275.0	327.1	163.5	122.0	150.0	44.8	22.4
1	100	772.0				91.0			
1	100	1362.0				182.0			
1	100	1505.0				180.0			
10	100	1573.0	1600.0	676.0	338.0	190.0	190.0	95.1	47.5
10	100	2450.0				313.0			
10	100	1438.0				144.0			
10	100	809.0				900			
				h2					
1	100	1616.0	1575.0	557.1	278.5	198.0	180.0	70.2	35.1
1	100	777.0				72.0			
1	100	1646.0				221.0			
1	100	2116.0				214.0			
10	100	3062.0	2525.0	436.3	218.2	366.0	400.0	21.5	10.7
10	100	2017.0				412.0			
10	100	2580.0				398.0			
10	100	2374.0				411.0			
				Rh					
1	100	1852.0	1975.0	249.8	124.9	276.0	280.0	16.3	8.2
1	100	2314.0				284.0			
1	100	1750.0				248.0			
1	100	1878.0				280.0			
10	100	2012.0	1975.0	191.9	95.9	303.0	310.0	28.8	14.5
10	100	2135.0				338.0			
10	100	1961.0				319.0			
10	100	1681.0				270.0			

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